

Stefan Furgyik

**ON THE PATHOGENESIS OF
SQUAMOUS CELL CARCINOMA
OF THE HUMAN CERVIX UTERI
EPIDEMIOLOGICAL AND EXPERIMENTAL
STUDIES**

Malmö 1984

Diss. Lund.
1984



Organization LUND UNIVERSITY Department of Obstetrics and Gynecology University Hospital S-214 01 Malmö, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue 1984 11 15	
		CODEN: LUMEDW/(MEKM-1010)1-124 (1984)	
Author(s) Stefan Furgyik		Sponsoring organization	
Title and subtitle ON THE PATHOGENESIS OF SQUAMOUS CELL CARCINOMA OF THE HUMAN CERVIX UTERI. Epidemiological and experimental studies.			
Abstract The woman's sexual behaviour is decisive in the etiology of cervical neoplasia (CN). Several factors are involved, but transfer of a carcinogenic agent from the man to the woman appears to be predominant. One-fourth of all women who have had a gonorrheal infection some 24-23 years earlier acquire preinvasive or invasive squamous CN, with a relative risk ratio of 4.6 ($p < 0.001$). The arithmetic mean interval between gonorrhea and carcinoma in situ is 11.6 years, and 13.5 years for invasive CN. The gathering of anamnestic information concerning gonorrhea is a simple task, suitable for the estimation of risk related to sexual behaviour. The role of the man in transferring the agent is confirmed by the increased occurrence of CN among wives of men who had gonorrhea before they had met their future spouse. 13.7% of women married with men previously infected with gonorrhea acquire CN; standard morbidity ratio 3.41 ($p < 0.0001$). These findings verify the connection between gonorrheal infection and CN. The occurrence of CN has been studied in near relatives of CN patients. Relatives of the patient's male partner constitute the control group. Among mothers of patients, CN was found in 7.9%, vis-à-vis 1.1% of the control group, and in sister, 7.5% vs. 1.1%. The results indicate a familial predisposition toward CN. Interaction between infectious agent and hormonal effect are inadequately known. For further studies, animal experiments will be suitable. It is difficult to successfully xenotransplant normal portio tissue, and preinvasive and invasive CN subcutaneously. When implanted into nude mice, normal and preinvasive portio tissues survive only to a limited extent and a grafting technique other than subcutaneous should be considered. HSV-2-related antigen in normal portio epithelium and cervical neoplasma can be demonstrated more often after grafting into nude mice. The increased expression of HSV-2 antigen may be due to the weak immune defence of the mouse.			
Key words Cervix neoplasm, Epidemiology, Neisseria Gonorrhoea, Familial&Genetics, Herpesvirus Hominis, Mice, Nude			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language English	
ISSN and key title		ISBN	
Recipient's notes		Number of pages 124	Price
		Security classification	

Distribution by (name and address)

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CARCINOMA OF THE HUMAN CERVIX UTERI.

Epidemiological and experi-
mental studies

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten
vid Lunds Universitet för avläggande av medicine doktors-
examen kommer att offentligen försvaras i Kvinnoklinikens
föreläsningssal, Allmänna Sjukhuset i Malmö, torsdagen den
15 november 1984 kl. 09.15.

av

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Malmö 1984

Translated by M. Brandt

Printed in Sweden

Team Offset, Malmö,

1984

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PUBLICATIONS

This thesis is based on the following publications:

- I Furgyik S and Åstedt B: Gonorrheal infection followed by an increased frequency of cervical carcinoma.
Acta Obstet Gynecol Scand 59:521-524, 1980.
- II Furgyik S.: Increased frequency of carcinoma of the uterine cervix in women married to men with a previous gonorrheal infection - an epidemiological study.
Acta Obstet Gynecol Scand (in press) 1984.
- III Furgyik S, Grubb R, Kullander S, Sandahl B, Wingerup L and Eydal A: Familial occurrence of cervical cancer, stages 0-IV.
Acta Obstet Gynecol Scand (submitted for publication, 1984).
- IV Furgyik S and Rausing A: Xenotransplantation of human cervix uteri into nude mice. Histological studies of transplants with normal tissue, and preinvasive and invasive squamous carcinoma.
Acta Obstet Gynecol Scand (submitted for publication, 1984).
- V Christenson B and Furgyik S: HSV 2-associated antigens in human cervical tissues before and after grafting in nude mice.
Acta Obstet Gynecol Scand 62:461-466, 1983.

In the text, these papers are referred to by their Roman numerals.

INTRODUCTION

No other gynecological cancer has over so many years attracted so much attention as squamous cell cervical neoplasia (CN)*. It is a fact that some types of women contract this type of cancer more often than others. In large materials has it been possible to discern that certain factors, certain types of behavioural pattern, occur more often in women with CN. There is much to indicate that CN is a sexually transmitted disease (STD). The woman contracts the disease, but it may be the man who acts as the transfer link, or as the bearer of the agent. However, it must be assumed that there are other factors that complicate the picture. It is probable that many women come into contact with the agent that causes CN, yet some escape its effects. We must assume that there are individual differences in the defence mechanism.

Attempts to solve some of the CN mystery have been made by conducting experiments on animals. They can help us by complementing or explaining clinical phenomena.

EPIDEMIOLOGICAL STUDIES

The question of virginity. As long ago as 1842, Rigoni-Stern (1842) observed an increased incidence of uterine cancer among married women, in comparison with cloistered nuns. Gagnon (1950) published his renowned investigation of medical records from several convents, covering a period of 20 years. In the records of 13,000 nuns he found not a single case of CN, though there were 12 cases of cancer corporis uteri. The low frequency of CN among nuns has been confirmed by Towne et al (1955), though he did find a few isolated cases. In an investigation of 146 nuns, 8% were found not to be virgins (Rotkin, 1981). Wynder et al (1954) reported that 1% of whites and negroes affected by CN stated that they had never experienced

* CN has been used for preinvasive or invasive squamous cervical neoplasia. Carcinoma in situ (CIS) has been used specifically for preinvasive cancer and invasiveness has been specified

coitus. Martin (1967) calculated a ratio of virgins/non-virgins with CN of 4:1,000 in the USA. For Swedish women, Kottmeier (1953) calculated the ratio to be 1:1,000.

Age at sexual debut. Low age at sexual debut for women is considered to involve an increased risk of contracting CN (Wynder et al, 1954; Rotkin et al, 1962; Stewart et al, 1966). It is considered that during adolescence the cervical epithelium is most susceptible (Rotkin, 1973). In investigations of women with coital debut before the age of 20 years and also before 17, both invasive and non-invasive CN were more common among the latter. The various published works are in close agreement (Wynder et al, 1954; Terris et al, 1960; Aitken-Swan et al, 1966). Rotkin (1973) found that the mean risk ratio on comparison with a control group was 1.5 with a coital debut before 20 years of age and 2.4 for those whose coital debut was before 17 years.

A comparison was made between 300 women with CN who admitted to having numerous premarital and extramarital sexual partners, versus a control group of 1,200 women (Singer, 1982). When age at first coitus was standardized, the previously significant difference between controls and women with CN with respect to multiple partners disappeared. In another work (Harris et al, 1980), the number of sexual partners was found to be related to sexual debut - the earlier the debut, the greater the number of partners. The risk is related to women without a partner or with one only and with sexual debut at 21 years or later. No increase in risk in connection with early sexual debut was found when the number of sexual partners was taken into account. Women with early sexual debut had CN diagnosed at a younger age than those with a later debut. This was true for both whites and negroes. In the case of a sexual debut before 16 years, the average age at diagnosis was 44 years. This contrasts with a corresponding diagnosis age of 54 years with debut after 25 years (Wynder et al, 1954).

Age at first marriage. Boyd et al (1964) compared age-matched patients with CN versus patients suffering from other diseases. In the CN group, 3% were unmarried and in the control group, 12.8%. As a rule, the CN women had married earlier than those in the control group. In the material published by Wynder et al (1954), whites and negroes with CN were considerably younger at first marriage than were the controls. 19% whites and 32% negroes had been married at the age of 16, vis-à-vis 5% and 13% of controls. According to Christopherson (1965) the highest frequency of CN is among women who marry before the age of 15. Similar findings have been reported by others (Lombard et al, 1950; Abou-Daoud, 1967).

Early age at first marriage is often combined with low socio-economic standard, often leading these women into a series of marriages, or a multiplicity of sexual partners. Such women run a greater risk of contracting a venereal disease, undergo more abortions, and give birth at a younger age than women married at a later age. Martin (1967) found at least one of these factors in the life history of 75% of women with CN, compared with 25% of controls.

Age at first pregnancy and partus; number of pregnancies and births. According to Wynder et al (1954), pregnancy does not influence the pathogenesis of CN. Other workers have come to the same conclusion (Rotkin et al, 1962; Boyd et al, 1964). Stewart et al (1966) found a close relationship between the age when a first pregnancy was terminated and the age at first coitus. Numerous pregnancies may be merely another expression of protracted sexual exposure, beginning at an early age (Moghissi et al, 1968). There was no increase in incidence of spontaneous abortions among women with CN, though there were more induced abortions (Rotkin et al, 1962).

Cervical lacerations occurring in connection with parturition, previously regarded as a risk factor for the

development of CN do not, according to more recent investigations, constitute any such risk (Jones et al, 1958).

The woman's sexual partner. Women affected by CN often have more than one sexual partner; two or three in 70%, vs. 48.5% of controls (Rotkin, 1967) and in another study 42.5% vs. 16.7% (Martin, 1967). In another work (Terris et al, 1960), 54% of CN patients admitted to having extramarital sexual partners, whereas only 26% of the controls did so. In yet another investigation, the strongest risk factor for cervical abnormality among 237 women was multiplicity of sexual partners. Women reporting six or more such had a risk 6.1 times as high as those with only one partner, irrespective of age at first coitus (Harris et al, 1980). The incidence of prostitution among the CN patients was four times that among controls with the same social level (Røjel, 1953). In a report of prison inmates with a large proportion of prostitutes, CN was six times more common than in a comparable control group (Pereyra, 1961). One of the highest reported rates is 92 of 1,000 women in a London gaol (Singer, 1982).

In epidemiological studies, however, it is difficult to obtain precise details about numbers of sexual partners.

The woman's sexual partner constitutes a risk factor if he himself has cancer of the penis. Penile cancer occurs most often in societies where circumcision is not practised and where penile hygiene is neglected (Riveros et al, 1963). In Puerto Rico, CN and penile cancer are both common. These two forms of cancer have one thing in common - both occur frequently in the lower social classes. The presence of CN among wives whose husbands had penile cancer was found to be 8-fold to that in a control group (Martínez, 1969). A significantly higher frequency of CN among women whose husbands had penile cancer was reported also from New York (Graham et al, 1979) and from Japan (Kurihara et al, 1956).

A man can evidently act as a vector between two women. In a carefully checked prospective epidemiological study, Kessler (1977) investigated the wives of men who had previously been married to women with CN. In 2.7% of second wives of these men, CN had occurred subsequently, vs. 1.1% of women in the control group. Altogether 14% of second wives contracted CN or abnormal cytological changes, vs. 8% of controls. Kessler calls these couples "marital clusters".

A man's occupation can also constitute a risk for his wife or sexual partner. In a large cytological material from a population screening program, Wakefield et al (1973) found a close correlation between positive or suspicious cytologic findings and the husband's occupation. The same correlation was also found between husband's occupation and presence of invasive CN. The lowest proportions of positive and suspicious findings, 4.11 per 1,000 were reported for the group "professional, technical workers and artists", and the highest proportions, 17.73 per 1,000 among wives of "labourers not classified elsewhere".

Socio-economic status. In one of the earliest comprehensive epidemiological works, a social difference was found in the incidence of CN (Kennaway, 1948). In all the more recent epidemiological investigations on CN that have considered the socio-economic status of the woman herself or that of her husband, the findings have been the same (Clemmesen et al, 1951; Devesa et al, 1980). Even poor nutrition and vitamin A deficiency have been considered as an explanation (Wynder, 1969). On the positive side, dietary rules among orthodox Jewish women have been put forward as protective factor against CN (Horwitz, 1927). Sociologists have demonstrated that there is a correlation between early sexual debut and social status; the poorer and less well educated the individual, the earlier the age at first coitus (Rotkin, 1981). Those low on the socio-

economic scale marry on average 2 years earlier and become pregnant 4 years earlier than those in the higher socio-economic brackets (Christopherson, 1965). There is thus a relation between early sexual debut, early first marriage and first pregnancy, and socio-economic status. The difference in frequency of CN between caucasians and negroes disappears when one takes social class as starting point (Christopherson et al, 1960).

Ethnic factors. The incidence of CN is noticeably lower in Jewesses. This observation has been confirmed by many researchers (Horwitz, 1927; Clemmesen, 1951; Wynder et al, 1954), also in a report from Radiumhemmet in Stockholm, Sweden for the years 1914-47 (Kennaway, 1948). By contrast, CN is common among the Negro population of the U.S.A. and among Puerto Ricans, where the average incidence and mortality rate are more than double that of white women (Cutler et al, 1981).

An explanation to the low frequency of CN among both Jewesses and non-Jewish women married to circumcised men and who have not indulged in extramarital relationships was thought to be just the fact of circumcision (Wynder et al, 1954). Nevertheless, among wives of circumcised Muslims, CN is just as common as among wives of the non-circumcised (Huber, 1960). Circumcision itself can therefore not account for the low incidence of CN among Jewesses.

Pereyra (1961) considers that the etiological factors are individual rather than racial and that, given the same opportunity for sexual exposure, both white and non-white women can prove equally susceptible to this disease.

Rural and urban factors. There is a difference in frequency of CN between rural and urban women (Berggren, 1957; Levin et al, 1960). In one investigation of 1,881 cases of CN in Japan (Segi et al, 1957) it was found that rural women had married younger than urban women, generally

speaking. Even so, the incidence of CN was higher among the urban women. Rural women gave birth at a younger age, yet urban women had more cases of CN, but gonorrhea was more common among the urban women.

An investigation of an island-dwelling population off the coast of Norway revealed only one case of CN among 8,000 inhabitants over a 9-year period. On the basis of the ratio for Norway as a whole, 7 cases would have been anticipated. All those factors that should have led to an increased incidence of CN - low socioeconomic status, early sexual debut or marriage, poor hygiene, and non-circumcised men - were present in this population. However, there were virtually no divorces and the occurrence of extramarital sexual intercourse was considered rare (Fjaertoft, 1968). The presence of venereal diseases was not studied in this work.

Heredity. Kennaway (1948) believes that "the genetic factor is obviously a possible one, but its action is difficult to prove or disprove". The literature contains case reports of CN in sisters (Bender, 1976) and in both mother and daughter (Andrews et al, 1981). In one investigation of 6,893 Danish twin pairs no discrepancy was found between monozygous and dizygous twins with regard to the incidence of CN (Harvald et al, 1963). Mey (1950) published a similar finding.

Recent studies have revealed, however, that there may be a genetic factor involved in cases of CN affecting high-risk women. "It seems as though in many cases of these women some deficiency occurs in the natural resistance of the cervix to the biological insults that it suffers" Singer (1982). He also found a significantly higher frequency of CN among women with deficient phenotypes as regards genetic protease inhibitor systems. It is remarkable that an over-representation of blood group A has been found in CN cases, at the expense of blood group O (Milsom et al, 1983).

Vaginal infections. Røjel (1953) found the same frequency of syphilis in prostitutes with CN as without CN and concluded that there must be a particularly virulent carcinogenic agent among prostitutes that has no relation to syphilis. *Trichomonas vaginalis* has been described in many reports as a finding with increased incidence in combination with CN, or dysplasia (Koss et al, 1959; Naguib et al, 1966), though nowadays it is considered to be merely an indication of sexual promiscuity without causal connection (Thomas, 1973). An anamnesticly increased incidence of gonorrhea in patients with CN has been reported by several investigators (Segi et al, 1957; Moghissi et al, 1968), and clientele at venereological clinics often have positive cytological smears (Greene et al, 1965).

A conceivable relationship between *Chlamydia trachomatis* and cervical cancer was pointed out by Niab (1970). Schachter (1975) isolated *Chlamydia trachomatis* from 4.1% of patients with cervical dysplasia, while Paavonen et al (1979) found the organism in as many as 17% of women with cervical dysplasia and 25% with invasive CN. Use of the immunofluorescence antibody technique revealed 77.6% of women with CN or dysplasia as having antibodies to chlamydia.

A higher frequency of Herpes simplex virus type 2 (HSV-2) antibodies was found among women with CN than among controls by Rawls et al (1968) and Nahmias et al (1968) and HSV-2 has been regarded as a potential etiological factor for CN. Experimental work has shown the oncogenic potential of HSV by its ability to transform hamster embryo fibroblasts (Duff et al, 1971). It has proved possible to demonstrate HSV-2 antigen by immunofluorescence technique in exfoliated cervical cells from CN patients and HSV-2 has also been isolated from cultivated cervical carcinoma cells (Aurelian, 1972). HSV-2-specific DNA and RNA has been demonstrated in invasive CN (Frenkel et al, 1972) as also has HSV-specific RNA in

cervical intraepithelial neoplasia II (McDougall et al, 1980).

A recent topic of interest has been human papilloma virus (HPV) infections (zur Hausen, 1976). HPV-antigen has been present in 72% of condylomatous dysplastic lesions, but in only 5.6% of non-condylomatous lesions (Syrjänen et al, 1982). There are several types of HPV, of which only a few can be connected with CN. Crum et al (1984) found HPV-16 cells with abnormal mitoses in flat cervical warts and considered that this type of wart is a precursor of invasive CN. The theory that both HPV and HSV-2 may be synergistic in the development of CN has been put forward by zur Hausen (1982). If certain cervical cells infected by papilloma virus were exposed to HSV, this could start a process of dysplastic cells leading to CIS and eventually to invasive CN.

Hormonal factors. No differences have been observed in menarche age, duration or amount of menstruation, menstrual disturbances, or age at menopause in connection with CN. Lombard et al (1950), however, found a significantly higher age at the onset of puberty in conjunction with CN. This could have been an effect of constitutional or environmental factors caused by poor health, malnutrition and poverty.

The uterine cervix is governed by hormonal regulation. Estrogen-binding protein is found in all parts of the cervix, but with the highest concentration in the region having a columnar epithelium (Sanborn et al, 1975).

Progesterone receptors have also been found (Rall et al, 1978). The concentration of estrogen receptors found in CN tissue has occasionally been increased (Evans et al, 1974).

Stern et al (1970) and Meisels et al (1977) found a higher frequency of cervical dysplasia in women who used oral contraceptives containing estrogens and progestagens, on comparison with other contraceptive means, though in

another work (Thomas, 1973) no such difference could be detected. Nevertheless the use of oral contraceptives is more widespread among women who had an early sexual debut (Meisels et al, 1977) and their use may therefore be an indicator of sexual activity. Both Harris et al (1980) and Vessey et al (1983) have shown that the risk of dysplasia or CIS increases in relation to the duration of oral contraception. No such tendency can be detected among users of IUDs.

Tobacco smoking. Smoking of 15 cigarettes or more per day increased the relative risk of contracting CN by 7.2% in a prospective study of 55,000 test subjects (Cederlöf et al, 1975). In cases of CIS, Thomas (1973) found "regular smoking" in 70% compared with 58% of a control group. Those whose "first child was conceived before marriage" and whose "first pregnancy was before age 20" smoked considerably more. Winkelstein (1977) considers it wrong to regard smoking as a sign of marital instability, but that smoking can be a significant risk factor. Women smoking 20 or more cigarettes daily ran a 3-4 times greater risk of getting CN than non-smokers (Harris et al, 1980). Buckley et al (1981) in his study on women who smoke, attempted to eliminate any possible risk that might derive from high-risk promiscuous men. Smoking remained a potent risk factor even after adjustment for sexual background.

EXPERIMENTAL STUDIES

Hormonal effects on tissue are highly complex. It is not known if, or in what way, hormones can affect an infectious agent and then how the agent may act in hormonally stimulated tissue (Kodama et al, 1983). Experimental studies may help us by complementing or explaining clinical phenomena.

HSV-infected mice had a higher mortality rate when progesterone was given, but also when females were pregnant

(Baker et al, 1978). When Forsberg et al (1976) produced cervical cancer in mice by administering 3-methylcholantrene, the incidence of squamous CN was found to be significantly higher among animals injected with both estradiol and prolactin, than in the controls or in animals injected with estrogen or prolactin alone.

The intensity of infection is greater and lasts longer in estrogen-treated guinea pigs experimentally infected with chlamydia than in the case of control animals (Rank et al, 1982).

Nude mice have been used as the experimental animal in studies of transplanted tumors (Povlsen, 1980). The background to these studies is that nude mice accept human tissue transplants, lack a normal thymus, have low lymphocyte counts and low immunoglobulin levels (Rygaard, 1981).

In the contributions to the discussion of the pathogenesis of human squamous CN so far, no unequivocal results have been published, despite the large number of investigations carried out. Those factors particularly suspected of involvement in the origin and course of CN, however, are infectious agents and hormones. The role of infection has been partially proved in epidemiological studies. For ethical reasons the influence of hormones is difficult to ascertain experimentally in women. One way of studying endocrine factors would appear to be to conduct xenotransplantation of CN tissue into nude mice.

AIM OF THE PRESENT INVESTIGATION

- to identify women running an increased risk of contracting CN, by means of information of previous gonorrhea (Paper I);
- to study the role of the male in transferring possible carcinogenic agents (Paper II);
- to establish whether CN occurs more often in close relatives of CN patients than in the control group (Paper III);
- to study the histological picture of normal portio tissue, and of preinvasive and invasive CN, both with and without hormonal stimulation in transplants to nude mice (Paper IV);
- to investigate how HSV-2 associated antigens behave during passaging of CN in nude mice, both with and without hormonal stimulation (Paper V).

MATERIAL AND METHODS

Paper I

Of 389 women with culture-verified gonorrhea in Malmö, 164 (42%) could be investigated 24-23 years later regarding the occurrence of CN. These subjects were still living in the Malmö area. The investigation can be regarded as representative, since the gynecological-cytological screening service in Malmö is extensive, covering 91.8% of all women up to the age of 69 that have been examined. All observed cases of CN have been verified histologically.

Paper II

In the same Malmö population, the presence of CN has been studied in 232 women who are or have been married to men who had a gonorrheal infection some 37-26 years earlier. The women were split into three groups: 1) those who were married at the time of their husband's gonorrheal infection and themselves were infected, 2) those who were married but escaped infection, and 3) those women who married the man in question later and who were not acquainted with the carrier at the time of his gonorrheal infection. If the men have been married more than once, every available wife and ex-wife has been checked for the presence of CN. For control purposes we used the incidence of all age-related CN cases in the same geographical area.

Paper III

In 180 consecutive patients treated for CN at the Department of Gynecology in Malmö during a one-year period, the anamnestic incidence of CN has been checked in first-degree relatives who were compared with a control group comprised of the husbands' first-degree relatives. In addition, the anamnestic incidence of gonorrhea in the patients has been charted. Blood group, genetic marker Gm(1) on the IgG molecule (Gm(1)) and secretory status were also determined.

Paper IV

Biopsy samples with normal portio epithelium (n=8), and preinvasive (n=14) and invasive (n=16) CN tissue were xenotransplanted into nude mice and the histological appearance of the implants was studied longitudinally.

Paper V

Normal portio epithelium (n=3), and preinvasive and invasive (n=7) CN tissue were xenotransplanted into nude mice. The occurrence of HSV-2 related antigen in the tissues was studied, both before and after the transplantation. Some of the animals were treated parenterally with estrogen or progesterone.

RESULTS

Paper I

Among 164 women who had had a gonorrheal infection 24-23 years earlier, CIS had occurred in 29 (17.7%) and invasive CN in 8 (8.3%). Altogether, CN had occurred in 37 women (22.6%) but in only 8 controls (4.9%). This implies a relative risk of 4.6 ($p < 0.001$) entailed by previous gonorrhea. The arithmetic mean interval between gonorrhea and CIS was 12.4 years for infected 15 - 20-year-olds, compared with 11 years for the remainder. CN was squamous epithelial neoplasm in all cases.

Paper II

Among 232 women married with men who had contracted a gonorrheal infection 37-26 years earlier, CN had occurred in 19/70 (27.1%) if the woman herself had contracted gonorrhea at the same time. CN had subsequently occurred in 5/23 (21.7%) even if the woman had not contracted gonorrhea from her husband, and in 19/139 (13.7%) if the woman had become acquainted with the gonorrhea-infected man only after the infection had been cured. According to the age-related incidence of CN in Malmö, there should have been 5.58 cases. This means a standardized morbidity ratio of 3.41 ($p < 0.0001$). All cases of CN were of the squamous epithelium type.

Paper III

CN had also affected the mothers of 14 (7.9%) out of 177 CN patients, vis-à-vis 1/103 (1.0%) of mothers in the control group. As regards sisters 20 years old or more, CN occurred in 14/188 probands (7.5%) vis-à-vis 1/93 (1.1%) in the control group, while the incidence of CN in mothers and sisters combined was 28/180 (15.6%) vis-à-vis 2/196 in the control group. There was no statistical significance regarding Gm(1), blood grouping or secretory status in these 180 cases of CN, compared with a normal population.

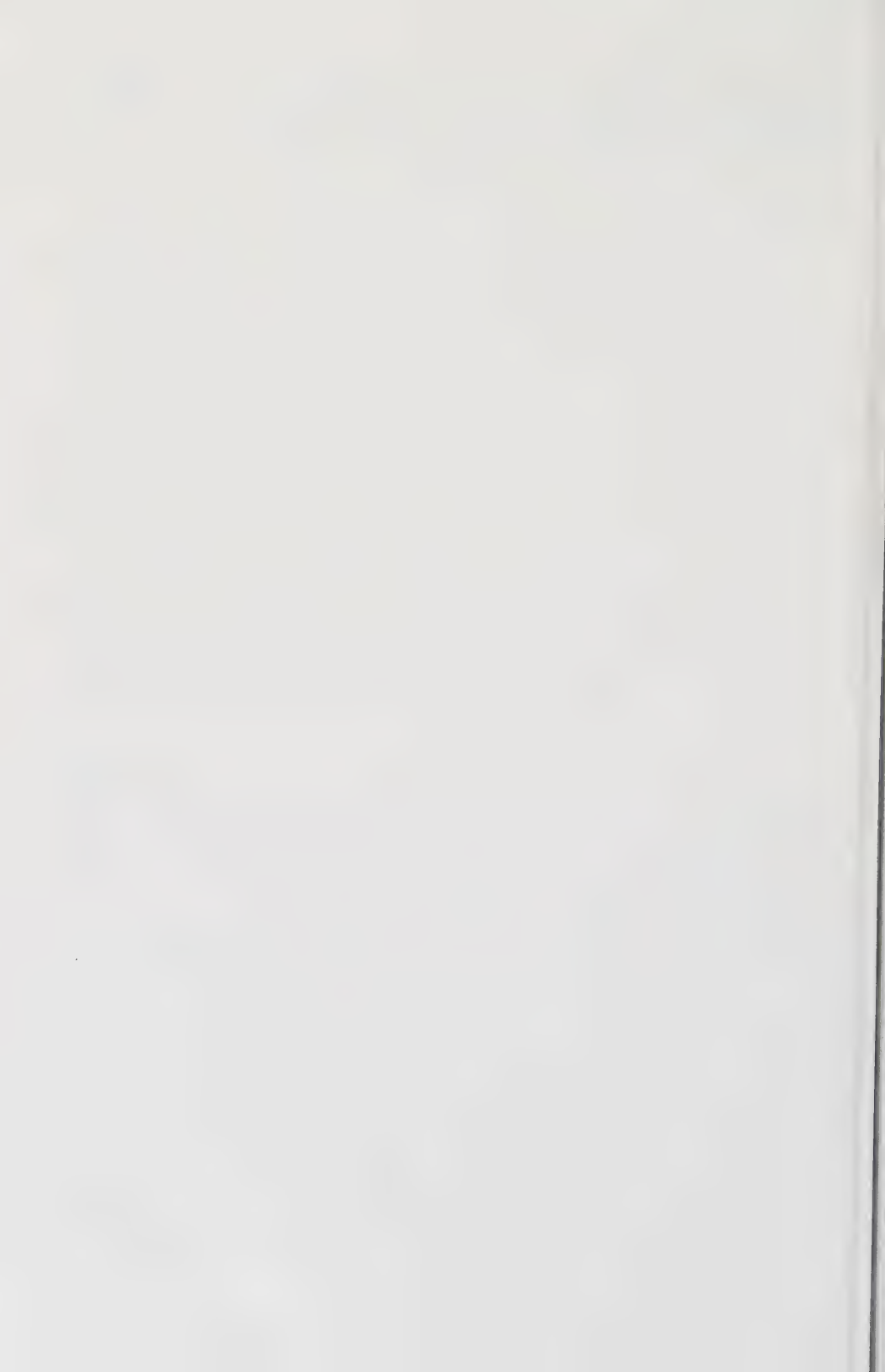
Paper IV

Biopsy samples of human porti were transplanted subcutaneously into nude mice. Three types of tissue were used: preinvasive and invasive squamous cell carcinoma and normal tissue. Eighteen animals were treated parenterally with estrogen or progesterone. Microscopically the transplants revealed degenerative changes in stroma and epithelium. Vital squamous epithelium was nevertheless observed up to the 10th postoperative week (normal epithelium), to the 8th week (preinvasive cancer) and to the 34th week (invasive cancer). In normal and preinvasive cancer epithelium, mitoses occurred until the 4th week. The stroma became hyalinized in some transplants, while in others it was invaded by murine vessels and after a time became merged with the mouse tissues. There were no differences in the appearance of transplants from estrogen-treated, progesterone-treated and untreated mice. The method is hampered by the degenerative changes in the transplant. In consequence it is difficult to evaluate the effects of medication or other manipulations.

Paper V

Normal portio tissue, CIS and invasive CN were implanted subcutaneously in nude mice and certain of them were injected with estrogen or progesterone. The presence of HSV-2 related antigen was analysed by means of anticomplement immunofluorescence before the implantation and then once a week for 4 consecutive weeks. Prior to grafting, HSV-2 related antigen could be demonstrated in 3 of 7 portio tissues with CN and in one of 3 women with normal portio epithelium. In the absence of hormone treatment, 5 out of 7 grafted CN tissue preparations reacted positively to HSV antigen. When normal epithelium was transplanted, it showed the same single presence of HSV-2 related antigen as before transplantation. During the period following grafting, HSV-2 related antigen was found in 6 CN tissues from 7 women and in 2 out of 3 grafts with

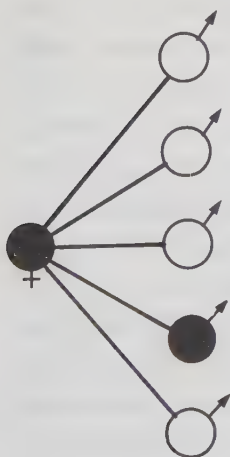
normal portio epithelium in estrogen-treated mice. Following progesterone treatment, no difference could be found compared with the finding before implantation.



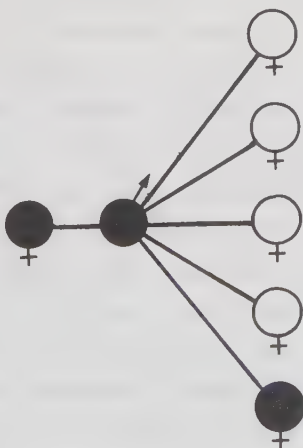
DISCUSSION

Earlier investigations have given us a sketchy picture of the epidemiology of CN. In many works the coital connection has emerged quite prominently. Virgins very seldom contract CN, whereas the more sexual partners a woman has, the greater is the risk of her contracting the disease. This relationship cannot be ascribed to a sheer mechanical effect, however. It seems more likely that CN is caused by an infectious agent passed from one partner to another as a STD. It can thus be assumed that some men are bearers of a carcinogenic agent(s). But the agent must have been acquired in turn from another source, i.e. from another woman in the form of a STD passed from that woman to the male carrier. The pattern as it evolves can be portrayed schematically as follows:

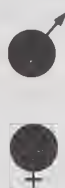
alternative 1



alternative 2



bearer of agent



Alternative 1 describes the case where the woman has had several partners, of which one or more may have been the bearer of the agent. According to alternative 2, even a woman who has had only one sexual partner can come into contact with the agent that causes CN.

Women who have several sexual partners run a greater risk of contracting a gonorrheal infection. That women with CN have a higher incidence of gonorrhea, established anamnesticly, has been known for some time (Segi et al, 1957; Moghissi et al, 1968). What was not known, however, is how the relationship would turn out in the long term. If one studies cervical changes simultaneously with an acute gonorrheal infection, one finds a low frequency, since CN develops very gradually and has a certain lag or "latency period" in relation to gonorrhea.

We proceeded from the assumption that carcinogenic agents can be acquired in conjunction with the gonorrheal infection and we therefore monitored women for 24-23 years after such an infection (I). We found that 22.6% of those previously infected by gonorrhea now had CN, vis-à-vis only 4.9% of the controls, which means a relative risk of 4.6 ($p < 0.001$). The youngest of the women who had had gonorrhea at an age of 15-20 years had probably not been able to come into contact with a carcinogenic agent much earlier than at the time of the infection in question. The arithmetic mean interval between gonorrhea and CIS was 12.4 years in the 15-20 years group, which may well correspond to the average time lag or "latency period" for the development of CN. Women who had gonorrhea when older had subsequently developed CN after a somewhat shorter interval, on average 11 years. Here one can question whether they had not in fact come into contact with an agent earlier and possibly even had a gonorrheal infection earlier too. Aside from this Dunn (1953) has arrived at the conclusion that in older patients transfer from CIS to invasive CN occurs in a shorter time. Pfleiderer (1981) considers this to be a primary other neoplasma in older women.

We found a close correlation between a history of gonorrheal infection and CN also in connection with a prospective familial investigation (III). In the 180 cases

of CN investigated, gonorrhea was recalled anamnesticly in 18.9%, but in invasive CN cases, in 26.3%. In the investigation concerned with the importance of the man's role in the transfer of a possible carcinogenic agent (II), it was found that 27.1% of women had CN 37-26 years after a gonorrheal infection.

It is difficult to obtain information on all sexual partners of a particular woman. As long as the male bearer is unaware of his condition, it is naturally impossible to conduct an investigation into the spreading of the infection. Kessler (1977) has studied "marital clusters", i.e. the occurrence of CN in both a previous and a present wife of the same man. This is one way of studying the man's role in the mechanism of agent transfer. Another indirect method has been used by the author (II). Proceeding from a previously established increased correlation between gonorrhea and CN (I), the incidence of CN in wives of men who had had gonorrhea was studied. 21.7% of women who did not become infected by gonorrhea from their husband later developed CN. It is interesting that of wives of men who had had a gonorrheal infection long before the two had any sexual contact, 13.7% of those women subsequently developed CN.

In a study of transfer of agents from men to women, one has to trace events far back in time. CN needs a certain "latency period" in which to develop. From gonorrheal infection to CN, this interval appears to be 12 years (I). In study II it was found preferable to trace even further back, starting from gonorrheal infection in young, unmarried men who first came into contact with their future wives much later. Moreover any latency period must be sufficiently long, i.e. sufficiently long time must be allowed to elapse after the couple have met. We have therefore traced back 37-26 years and this has proved adequate.

A woman who marries the man in question long after his gonorrheal infection and then acquires CN constitutes to

an increased degree an indication that the man was a bearer of the agent (virus?) leading to CN long after the gonorrheal infection.

When recording familial incidence of CN and the sexual anamnesis (III), we came across occasional women who had contracted CN even though they had had only one partner, according to their own statement. In one such case we could trace the man's contact with a woman who had had CIS.

The occurrence of CN in virgins is difficult to explain. One well documented case (Mogaji, 1973) of invasive CN was a married woman with an intact hymen. The woman's husband may have been the bearer of the possible agent. As it is known that sperms can lead to pregnancy even after ejaculatio ante portas (confirmed by own unpublished observation), it is not inconceivable that a possibly infectious agent can reach the cervix in the same way.

Multiple sexual partners and venereal diseases have been observed to an increased extent among patients with prostatic cancer (Steele et al, 1971) and an increased risk of CN was reported among spouses of prostatic cancer patients (Feminella et al, 1974). It is therefore conceivable that a sexually transferred agent may be the etiological link also in cancer of the prostate.

Age at sexual debut is considered to be the most important risk factor for CN, though in one work (Harris et al, 1980) the relation to the number of sexual partners is regarded as being even more telling.

We have conducted an anonymous anamnestic investigation concerning sexual debut and numbers of sexual partners (unpublished) in connection with cytological mass screening in Malmö, covering women 35-44 years old. The questionnaire was presented to women who had previously had at least two negative vaginal smears free from signs of atypia, and also to women with previous CN, confirmed histopathologically. The age range 35-44 years was chosen because of the minimal risk of finding CN in women with

two previous negative smears in this age group. The results indicate that women with early sexual debut have several partners and become pregnant earlier than those with a later debut. This applied to the entire investigation material. There was a higher frequency of early debut and several partners in the group that contracted CN. Among women with a late debut it was a more common finding that those with several sexual partners also had CN. Gonorrhea was found anamnesticly in 24.7% of women who had had CN, vis-à-vis 4.8% of the controls.

The number of sexual partners appears to be the factor implying the greatest risk of transfer of a specific agent. An increased risk of contracting CN, associated with the husband's occupation, has been proposed by several workers (Wakefield et al, 1973; Beral, 1974). However, this may be another way of expressing socio-economic status. Moreover, the occurrence of CN is appreciably more common among wives of men whose occupation takes them away from home and gives them greater possibility for extramarital relationships. Buckley et al (1981) found a relative risk of 7.8 for women whose husbands had had 16 or more extramarital relationships.

The differences in frequency of CN between different ethnic groups, found in earlier investigations, may be regarded as consequences of differences in ways of life, including factors that promote increased promiscuity.

If it had been possible to acquire cervical neoplasia by direct heredity, one would presumably have found an occasional case among the 13,000 nuns followed for 20 years (Gagnon, 1950). Even so, there has long been a suspicion that CN affects certain predisposed women. Evidently not all women who come into contact with the agent acquire the condition. CN was found in two of three wives of a man married three times, where the wife with normal cervical status was number 2 in chronological order (II). This condition might indicate that the man was the carrier of an agent that eventually caused CN in two of

the women, but not in the third. A predisposition can be determined genetically, possibly on an immunological basis. Other factors that can be "inherited" are living habits and environment, which may also predispose to the development of CN (III).

Whether steroids and contraceptives can influence the course of CN and if so, in which way, has not yet been established. Several investigations have produced results that may indicate a connection between steroid contraceptives and the origin of CN (Harris et al, 1980; Vessey et al, 1983). If this can be proved, it would be conceivable that this occurs by interplay between the CN agent and a hormone-influenced cervical epithelium. The portio epithelium might under certain circumstances be predisposed to accept an agent or to promote a quicker progress from dysplasia to CN (Stern et al, 1977).

Just as obscure as the question of a development of malignant cervical changes governed by hormonal contraceptives is the problem of whether pregnancy may affect cervical malignancy. If there were any such effect, one would have to assume a qualitative change in the epithelium when the agent is already present.

Simultaneous with the grafting of normal and neoplastic portio tissue, we have also implanted in some nude mice human placental and amniotic membrane tissue at another site, in the hope of simulating the hormonal conditions prevailing during pregnancy. Placental and amniotic transplants remained vital and produced hormones for 2 months. HCG levels in mouse blood could be monitored and mouse vagina demonstrated an intense estrogen stimulation (Boltenberg et al, 1984). The hormonal effect on transplanted portio epithelium and CN tissue could not be observed, however, which may be due to degenerative changes.

The possible connection between cigarette smoking and CN is one of the more recent epidemiological observations. Various hypotheses for the biological mechanism have been

put forward, including a direct effect of nicotine on the epithelium (Clarke et al, 1982), but no proof in support of any of these theories has been forthcoming. Barons (Baron, 1984) on the other hand pointed to the "anti-estrogenic effect" of smoking, which can lead to a low frequency of breast cancer, early menopause and postmenopausal osteoporosis.

HSV-related antigen was demonstrated more often following transplantation into nude mice (V). But it was not possible to decide if the HSV antigen had been made to reveal itself by estrogen treatment, or merely by transplantation into the host animal with low immunoglobulin levels. If the latter can evoke greater viral activity, it may be possible to prove the theory of the etiological role of the virus and confirm that there are differences in immunological defence among women. This need not apply only to HSV antigen but even to other viral agents. It has been observed that the altered immunology in connection with iatrogenic immunosuppression in renal transplantation gives rise to an increased incidence of CN (Schneider et al, 1983) which may strengthen this assumption. Continued studies should be more rewarding when we can ensure better survival of normal portio tissue and CIS in longitudinal studies.

In subcutaneous xenotransplantation it is difficult to get primary tumor of invasive CN to grow reproducibly (IV). Normal portio epithelium has already been made to grow, but only a few cases have been investigated (Eichholz et al, 1981). CIS has not previously been transplanted.

Our current investigation (IV) has shown that normal portio epithelium and CIS epithelium remain vital for a limited period. One explanation for this may be that portio epithelium, like epidermis, does not have the best qualifications for successful subcutaneous implantation. In support of this, one can demonstrate the better survival of transplanted metastatic invasive CN. It may be that

this cancer is already adapted to a different growth environment. Representative tissue for transplantation of CIS is nowadays difficult to obtain. One seldom finds a large enough single, kolposcopically visible area of CIS that can be removed in a suitable way in order for implantation.

For transplantation of normal and premalignant portio epithelium, one can try another localization in the mouse. For instance, one can sew portio mucous membrane into the mouse's back. It would be of value to obtain a reproducible experimental model with implanted normal portio and CIS, in which one could observe the structure and even therapeutical effects such as from cytostatics and hormones.

CONCLUSIONS

- 1) Women who at some time have had a gonorrheal infection develop cervical neoplasia (CN) to an increased extent. To assess the risk, the collecting of anamnestic information is a simpler and more suitable method than using information on sexual behaviour.
- 2) An increased incidence of CN is encountered in women who marry men who have previously had gonorrhea. This confirms the man's role as bearer and vector of a carcinogenic agent connected with CN. The man can be the bearer of this agent for a very long time.
- 3) Women with CN and women with a normal portio who have lived with the same man would appear to be suitable candidates for pathogenetic and immunological studies.
- 4) There is a familial disposition to CN. Environment and way of life can also leave their impression.
- 5) The subcutaneous xenograft implantation of normal portio tissue and preinvasive CN into nude mice does not appear to be a suitable method for longitudinal studies. Another grafting technique than subcutaneous should be considered.
- 6) HSV-2-related antigen in CN and normal portio tissue could be demonstrated after xenografting in nude mice when they were subsequently treated with estrogen. The increased expression of HSV antigen may be due to the weak immune defence of the mouse, and/or to the hormonal treatment.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to the following:

Professor Stig Kullander, Head of the Department of Obstetrics and Gynecology, University of Lund, General Hospital, Malmö for new ideas, for giving encouragement, and for constant interest;

Associate Professor Alf Rausing, Department of Pathology, University of Lund, at the General Hospital, Malmö, for help and support throughout the investigations;

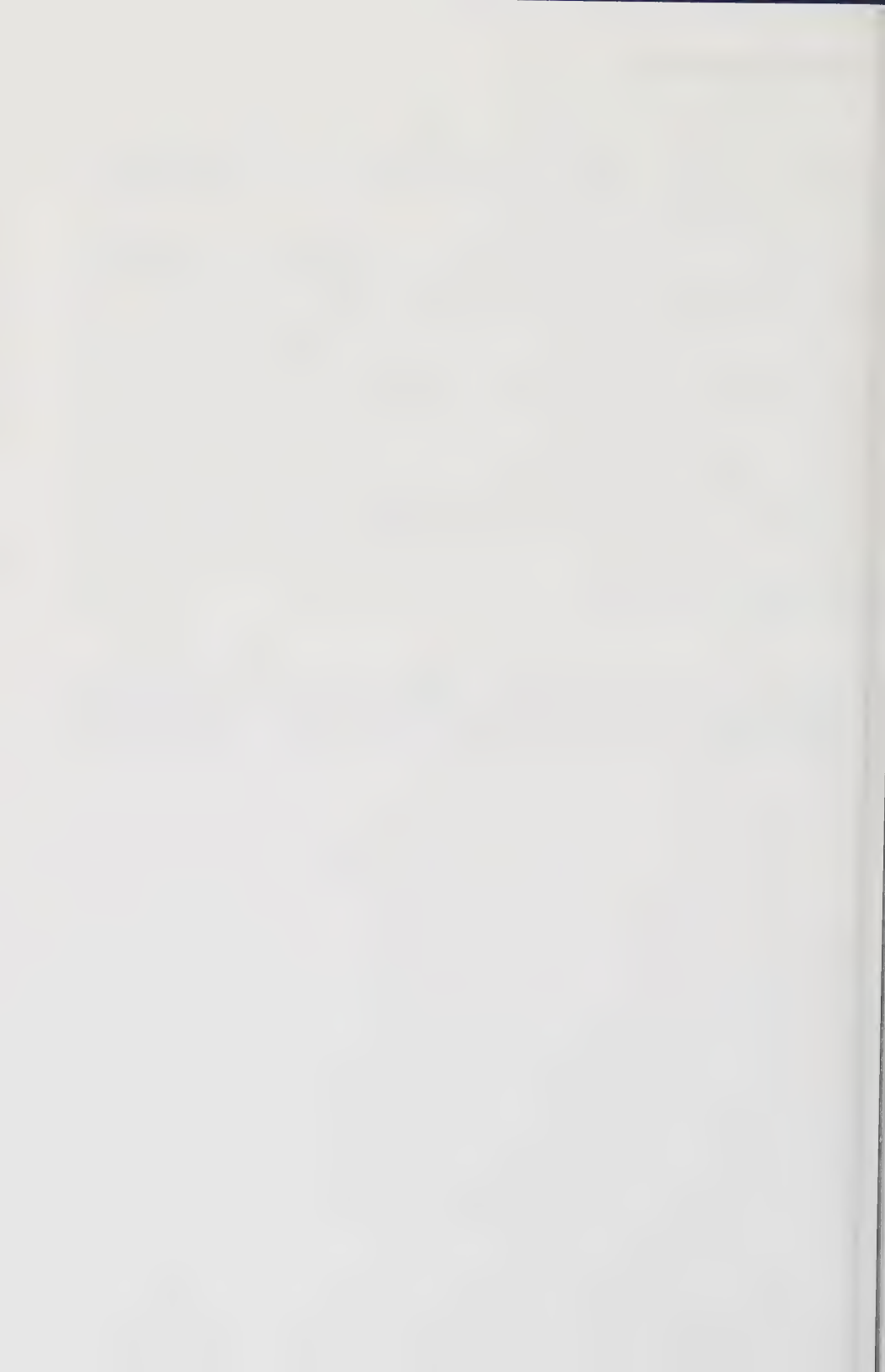
Associate Professor Bengt Bjerre, Department of Obstetrics and Gynecology, University of Lund, General Hospital, Malmö, for critical reading of the manuscript;

Miss Agneta Enander for skilful help with the mice and other technical assistance;

Mrs Agneta Norén and Miss Ann-Christin Flyving for careful preparation of the manuscript and excellent type-writing;

Mrs Inga Green for kindly helping with the cytologic screening material.

This work was made possible thanks to financial assistance from the Sigrid Simonsson and Agni Ohlsson Foundation, Trelleborg, for which the author expresses sincere gratitude.



GONORRHEAL INFECTION FOLLOWED BY AN INCREASED FREQUENCY OF
CERVICAL CARCINOMA

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Abstract. In a town with a fairly centralized health service and a well organized gynecologic health control, women who had had gonorrhea in 1954 or 1955 were reviewed for presence of cervical neoplasia and compared with age-matched controls. Of 164 women studied, 29 (17.7 per cent) had 24 or 23 years later developed cancer in situ, compared with 7 (4.3 per cent) of the controls ($p < 0.001$), while 8 (4.3 per cent) had invasive cancer colli uteri against only one (0.6 per cent) of the controls ($p < 0.02$). Malignant disease of the portio proved at least four times as common among the women with gonorrhea in their history as among the controls. The findings corroborate the view that cervical carcinoma is a sexually transmitted disease.

It may be assumed that at least every fourth woman who had had gonorrhea had been or is a carrier of the carcinogenic agent(s). It is probably easier to search for and detect such agents in association with gonorrhea than in patients with already manifest cancer of the uterine cervix.

INTRODUCTION

Sexual intercourse is thought to play an important role in the development of cervical carcinoma. The disease is also more common among married women (5). It is rarely diagnosed in nuns (3). Its frequency is increased among women who had their first pregnancy early in life (8) and among women who started their sexual activity while still relatively young (16). Sexual promiscuity (15), prostitution (13) and low socio-economic status (6) are other factors related to an increased frequency of carcinoma of the cervix. Further evidence of sexual intercourse being involved in the causation of the disease is the relatively high frequency of vaginal infections in such patients. The frequency of cervical carcinoma is reported to be increased in women who have or have had venereal disease (4,9,10,14). In England, Scotland and Wales a covariation has been demonstrated for frequency curves of cervical carcinoma and venereal disease in two cohorts 20 years apart (1). The frequency of cervical carcinoma of the cervix is also reported to be high in marital clusters (7). The carcinogenic agent common in these studies is not known. Herpes virus (12) might be responsible but so too could be carcinogenic metabolites of bacterial origin.

The present study was designed to get an idea as to how to trap the carcinogenic factor and to elucidate the latency period between infection and development of cervical cancer. We therefore reviewed women who had had gonorrhea 23 or 24 years previously and been repeatedly examined cytologically since then. The occurrence of preinvasive and invasive cervical carcinoma was recorded and compared with the corresponding figures in closely age-matched controls.

PATIENTS AND METHODS

The material consisted of the records of 164 (42.2 per cent) of 389 women with gonorrhea who had been seen at the Department of Gynecology or Department of Venereal Diseases-

es, Malmö General Hospital, in 1954-55 and who were still living in the area of Malmö which is served by this hospital only. In all of them the diagnosis had been confirmed by culture of material from the uterine cervix, urethra, and sometimes from the rectum. From 1956 they had been followed cytologically either by gynecologic practitioners or at the Department of Gynecology at the same hospital. From 1966 they had taken part in mass screening examinations every fourth year. When the vaginal smears showed changes, biopsy specimens had been obtained by means of the colposcope. The diagnosis of carcinoma of the cervix was always verified by histologic examination of biopsy specimens or of specimens obtained at conization or hysterectomy.

The controls consisted of 164 closely age-matched women who had been examined with cytological smear technique in the same way as the patients with gonorrhea in their history. The frequency of gonorrhea in the control group was calculated to be 0.5-1.0 case according to the statistics for venereal disease in Malmö.

Fisher's exact test was used in the statistical analysis.

RESULTS

Carcinoma in situ had developed in 29 (17.7 per cent) of the 164 women who had previously had gonorrhea, compared with 7 (4.3 per cent) of the controls ($p < 0.001$). Invasive cervical carcinoma was diagnosed in 8 (4.9 per cent) of the patients, against one (0.6 per cent) of the controls ($p < 0.02$). Preinvasive or invasive carcinoma was thus demonstrated in 37 (22.6 per cent) of the patients, but in only 8 (4.9 per cent) of the controls (Table I), which implies a relative risk of 4.6 ($p < 0.001$). All the invasive tumors of the cervix were squamous epithelial lesions. No adenocarcinomas were seen.

Table I. Preinvasive and invasive cervical carcinoma in women who had had gonorrhea 24 or 23 years previously (n=164) compared with controls (n=164)

Stage	Patients	Controls
Ca in situ	29 (17.7)*	7 (4.3)
Invasive ca colli uteri	8 (4.9)	1 (0.6)
Total	37 (22.6)	8 (4.9)

* Figures within parentheses denote per cent

The age distribution of the patients at the time of infection with gonorrhea and at the time of diagnosis of carcinoma is given in Table II. The frequency of gonorrhea was highest in the 21-25 age class. The youngest patient was 15 years old at the time of the diagnosis, and the oldest, 46. The ages of the patients with carcinoma in situ ranged from 26 to 60 years (mean 35) at the time of the diagnosis. The corresponding figures for the controls were 29 to 56 (mean 37). Invasive cervical carcinoma was diagnosed in patients between 35 and 60 years (mean 44). The only control who had this advanced stage was 41 years at the time of diagnosis. Of the 8 patients with invasive cervical carcinoma one was in stage 1a, 5 in stage 1b, one in stage 2b and one in stage 4. In the control group the single case of invasive carcinoma was in stage 1 b.

Table II. Please see page 54.

Fig. 1 shows the interval between the time of infection with gonorrhea and the diagnosis of carcinoma in situ or invasive cervical carcinoma. The arithmetic mean for carcinoma in situ was 11.6 years and for invasive carcinoma, 13.5 years. One 51-year-old patient in whom a Papanicolaou smear suggested further investigation was not

INTERVAL BETWEEN DIAGNOSIS OF GONORRHOEA AND CARCINOMA IN SITU OR INVASIVE CERVICAL CARCINOMA

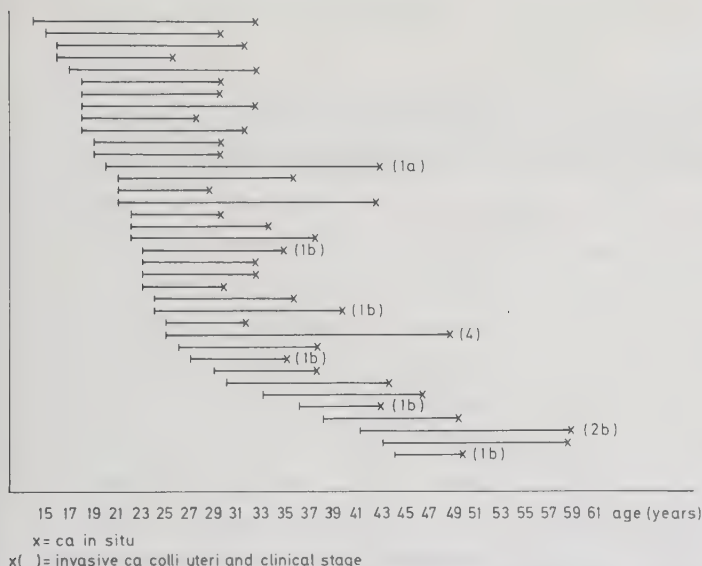


Fig. 2 shows the intervals between the diagnosis of gonorrhea and of carcinoma in situ or invasive carcinoma, regardless of age of the patients. The shortest interval for carcinoma in situ and for invasive cervical carcinoma was 6 years, the longest 21 and 23 years, respectively. The curve shows a substantial increase after 11 years. After additional 5 years the frequency of new cases of carcinoma in situ decreased, while that of invasive carcinoma did not.

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shown changes which were only temporary, however, or which further investigation had shown to be benign. The corresponding figures for the control group were 2 and 15 women.

INTERVAL BETWEEN DIAGNOSIS OF GONORRHOEA AND CARCINOMA IN SITU OR INVASIVE CERVICAL CARCINOMA IRRESPECTIVE OF AGE

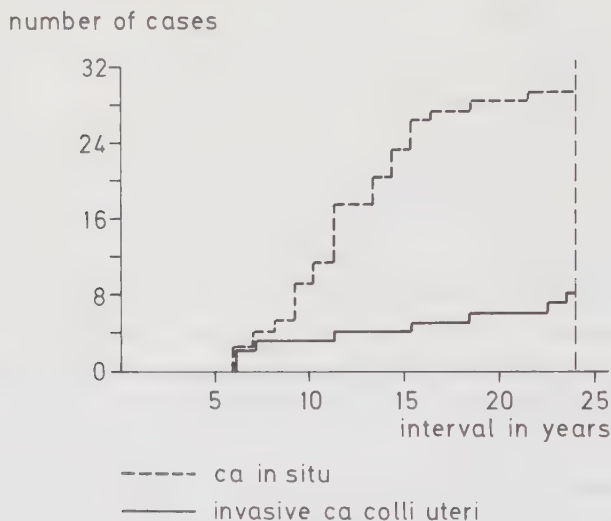


Fig. 2 Interval between diagnosis of gonorrhea and carcinoma in situ or invasive cervical carcinoma irrespective of age.

DISCUSSION

The investigation corroborated the finding in earlier retrospective investigations that cervical carcinoma is more common among women with vaginal infection in their history. The present study can be considered as a prospective investigation of a well defined clinical material of patients with gonorrheal infection diagnosed at Malmö General Hospital in 1954 and 1955 by culture of material from the cervix, urethra, and sometimes also from the

rectum. When starting retrospectively with women in various stages of cervical carcinoma, anamnestic data about venereal disease are of doubtful value and are often denied by the patients.

Of the primary material of 389 patients, 164 (42.2 per cent) were still alive and residing in the catchment area of the hospital in 1978, when they were reviewed for preinvasive or invasive cervical carcinoma. There is no evidence of any significant difference in general health between the women still living in Malmö and the remainder who had moved from the catchment area and were therefore not included in the material. It is possible, however, that the gynecologic screening of as many as 91.8 per cent of all women up to 69 years of age in Malmö (2) might have resulted in a higher frequency of early diagnosis of cervical carcinoma than elsewhere. This possibility is strengthened by the coincidence of the rise of the curve in Fig. 2 in the 11th year after infection with gonorrhea, simultaneous with the inception of the screening project.

The controls consisted of closely age-matched women who had participated in the screening in exactly the same way as the women known to have previously had gonorrhea. Since we were unaware whether the 164 controls had previously had gonorrhea or not, they may be regarded as a representative sample of the general female population of the town with an anticipated negligible incidence of 0.5-1.0 case in this group.

As long as we do not know the causal factor of carcinoma in situ, or of invasive cervical carcinoma, we prefer to use the term "interval" instead of latency period for the time between the diagnosis of gonorrhea and the diagnosis of malignant changes in the portio.

We cannot say anything about the actual causal agent of the malignant process, i.e. whether it could be *Neisseria gonorrhoeae* alone or *N. gonorrhoeae* combined with some other simultaneously acquired infectious agent of bacterial or viral origin (11). Furthermore a carcinogenic factor

acquired at the same time as the gonorrheal infection cannot be excluded.

Assuming that the causal agent that started the malignant process was acquired in association with the gonorrhea, the youngest group is the most interesting. In view of their age they could hardly have been exposed to cancerogenic agents for any length of time before they had been infected with *N. gonorrhoeae*. This would mean that in this age class the interval (12.4 years) between the diagnosis of gonorrhea and the detection of carcinoma in situ or invasive carcinoma might be a fair estimate of the latency of such lesions.

The arithmetic mean interval between gonorrhea and carcinoma in situ was 12.4 years in the 15-20 years group, compared with 11 in the remaining patients, i.e. a difference of only 1.4 years. This difference might be explained by genetic lability with increasing age or previous, unknown infections. Women who acquired the infection later in life might have been exposed to some causal agent of the malignant process earlier in life. If so, it would explain the shortness of the interval between the diagnosis of gonorrhea and of preinvasive or invasive carcinoma of the cervix in such women. Furthermore, one cannot exclude the possibility of their having had gonorrhea even on some still earlier occasion.

It was not thought worthwhile to study the effect, if any, of parity or socio-economic status, as such an investigation would have been very laborious and the results would hardly have warranted any clear-cut conclusion, for two reasons; first, substantial changes in socio-economic status during the long period covered by the present study, and second, the increasing frequency of cohabitation. We do not believe that prostitutes were over-represented in our material, but rather that it is an acceptable sample of urban women with gonorrhea in their history.

Herpes genitalis and/or other genital infections which may occur alone or combined with gonorrhea may be of importance in the development of malignant lesions. Vaginal smears from a group of 37 women in our hospital who had 1-5 years earlier had virologically verified herpes genitalis without coexisting gonorrhea were examined for cervical neoplasia. Cytologically, 35 of the women had a Papanicolaou smear I and 2 a Papanicolaou smear II. In both cases biopsy showed benign atypia (17). This does not mean that herpes is of no noteworthy importance, but the shortness of the interval precludes any definite conclusion as well as any comparison with women who had previously had gonorrhea.

The present results clearly show that the risk of developing malignant lesions in the portio was four times as high in women who had had gonorrhea as in those who had not. This increased risk is sufficient to warrant particular attention at the gynecologic examination of such women.

The similarity in age distribution regarding the occurrence of cervical neoplasia between patients and controls suggests that gonorrhea is associated with a carcinogenic agent but less likely with a promoter agent.

In search for the carcinogenic agent of carcinoma of the uterine cervix, one might expect a better chance of finding this agent at the time of infection than when a carcinoma of the uterine cervix has already reached a preinvasive or invasive stage.

ACKNOWLEDGEMENTS

We are grateful to Bo Gullberg, Institute of Statistics, University of Lund, for valuable help with the statistical analyses.

Table II. Age of patients at diagnosis of gonorrhea and of patients and controls at diagnosis of cervical neoplasia

Age groups	<15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-60	Total
Gonorrheal infection	1	45	66	26	8	8	7	3			164
Ca in situ				10 (2) *	9 (2)	5 (1)	2 (1)	2		1 (1)	29 (7)
Invasive ca colli uteri				2		1	2 (1)	2		1	8 (1)

* Figures within parentheses denote controls

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INCREASED FREQUENCY OF CARCINOMA OF THE UTERINE CERVIX IN
WOMEN MARRIED TO MEN WITH A PREVIOUS GONORRHEAL
INFECTION

An Epidemiological Study

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Abstract. An earlier investigation showed an increased frequency of squamous epithelial carcinoma of the cervix in women who had experienced gonorrhea, but that there was a long lag between the infection and diagnosis of the cervical malignancy. The present work is a retrospective study of cervical malignancy in women married to men who had had gonorrhea between 26 and 37 years previously. The women have been distributed into three groups: i) those who were married to their spouse at the time he had gonorrhea and who themselves became infected, ii) those who were married to their spouse at the time of his gonorrhea but who did not themselves become infected, iii) those who were not married to the man in question at the time of his gonorrhea and who do not recall having had had gonorrhea.

The incidence rates of cervical neoplasm were found to be: group i, 27.1%, group ii, 21.7%, and group iii, 13.7%. For all three groups the figures are significantly higher than in an age-related control group drawn from the normal population.

The results of the investigation lead one to suspect that a carcinogenic agent had been transferred by sexual intercourse together with the gonorrhea and that the man was the bearer of this agent long after he had undergone treatment for his gonorrhea, but that not all the women so exposed had developed a cervical neoplasm.

INTRODUCTION

The relationship between women's sexual behavior and the development of cervical neoplasm has been investigated many times previously. Early sexual debut (16), early first pregnancy (9), prostitution (11), promiscuity (14), low socioeconomic status (4), and genital infections (7) are all factors believed to be of significance in the development of cervical carcinoma. The absence of the disease in nuns (6) is confirmation of its sexual character. Possible transfer of a carcinogenic agent from men to women has previously been discussed (1,10,12,13). A higher frequency of the disease has been reported in women whose husbands' previous wives have had a cervical neoplasm (8). Even a husband's occupation has been connected with an increased frequency of cervical neoplasm (15). The carcinogenic agent is not known, but there is much to imply that cervical neoplasm is a sexually transmitted disease (2).

The study of sexual relations is a very complex matter. Respect for the patient's integrity means that one cannot always trace possible epidemiological connections in the way that one would wish. The sufferer's obligation in a case of gonorrhea to report sexual contacts can, however, occasionally add one more link to the chain of relationship.

A previous investigation has shown that gonorrheal infections are associated with cervical neoplasm (5). The rate of development of cervical neoplasm in women who had had gonorrhea 23-24 years earlier was found to be 22.6%, compared with 4.9% in an age-matched control group. This means that one can use gonorrheal infection as a suitable yardstick with which to follow up sexual contacts in the study of its epidemiological relationship to cervical neoplasm.

The present study used as a basis men who had had a gonorrheal infection and investigated the extent to which cervical neoplasm had occurred among their wives.

MATERIAL AND METHODS

The study was conducted in Malmö, a town in southern Sweden having about 230,000 inhabitants. Investigation was made of 772 men who, during the period 1944-55, had received a diagnosis of gonorrheal infection, based on culture from the urethra. Some 26-37 years later, 289 of these men were still living in Malmö and their civil status could be charted. It appeared that 172 (59.5%) had been married once, 75 (25.6%) twice, 8 (2.8%) three times, 3 (1%) four times, and 3 (1%) five times. Twenty-eight (9.7%) had never been married. Two-hundred and sixty-one men had thus been married at some time with altogether 373 women.

In Malmö in 1956, vaginal cytologic screening was introduced, followed in 1966 by mass screening for all women between the ages of 20 and 69 years (3). The cytological specimens have been assessed by the same cytological laboratory and the investigation of cytological changes has been carried out according to the same principles, with colposcopy and biopsy specimens in indicated cases. A diagnosis of cervical neoplasm was always confirmed by histological investigation. Ninety-two per cent of all women up to the age of 69 years have participated in the cytological mass screening or else had a smear taken by a privately practising gynecologist. Since there are local variations in the intensity of the cytologic screening in the country and consequently also differences in the diagnosis of cervical neoplasm, the material has been limited to women resident in Malmö. The evaluation was based on anamnestic information and records at the gynecological, cytological and histopathological departments. Checks were also made at the Cancer Registry of Sweden. Of the 373 women mentioned above, 58 (15.5%) were not resident in Malmö and had not been included in the screening program described above. Three of these 58 women had had carcinoma in situ (CIS) but, since no systematic

investigation was possible, none of them is included in the material.

In order to accomplish the statistical evaluation of the material it was found necessary to exclude 63 (20%) women from the rest of the material: 20 (6.3%) had never undergone a gynecological examination, a further 5 (1.6%) had not been examined cytologically during the past 10 years - the latest vaginal examination had taken place between 11 and 19 years previously. All these women were called to a gynecological health check but none had responded. Nine (2.9%) women had married the man in question in 1972 or more recently, and were therefore excluded. Fourteen (4.4%) were separated from the man before he acquired the gonorrheal infection and were therefore also excluded. Fifteen (4.8%) had had either mild or serious dysplasia evident in their latest vaginal smear but had not completed the recommended investigation. A further 20 (6.3%) women formerly resident in the municipality had died of other illness.

The remaining 232 women who are or have been married with men who have experienced a gonorrheal infection, were split into three groups. Group i was comprised of those who had had a gonorrheal infection at the same time as their husband, where the woman might have infected her husband, or vice versa. No subgrouping according to infection source was made. Group ii consisted of those who were married at the time their husband had his infection, though they themselves were not affected. Group iii is comprised of women who, according to the anamnesis, had never had a gonorrheal infection. The women in this group had not become acquainted with their future husbands until after the latter had had their gonorrheal infection (Table I).

The material was stratified by age and analysed by the Mantel-Haenszel χ^2 -test. Comparison of carcinoma cases, observed and expected, was carried out in terms of the standardized morbidity ratio (SMR) and the p-value was

calculated according to Poisson distribution. Statistical significance means a p-value of less than 5%, all values being calculated by a two-tailed test.

RESULTS

Among 70 women in Group i where both spouses had had a gonorrheal infection, CIS was found in 17 women and invasive squamous epithelial cancer of the cervix in 2, i.e. in 27.1%. Of those 23 who had been married at the time of their spouse's gonorrhea, but who themselves had not become infected (Group ii), 5 (21.7%) have developed CIS. In those women who had never had gonorrhea and who met their future spouse subsequently (Group iii) CIS was demonstrated in 19 cases out of 139 (13.7%). A statistically significant difference is evident ($p=0.0162$) between groups i and iii, but not between i and ii, or between ii and iii.

The incidence of cervical malignancy in women in Group iii has been compared, using data from the Cancer Registry for Southern Sweden, with the age-related incidence in the general population of Malmö municipality. The anticipated number of cervical malignancies calculated according to the general incidence would be 5.58. The 19 cases of cervical malignancy in the women who had married a man who had had a gonorrheal infection gives, however, a SMR of 3.41 ($p<0.0001$).

One woman with invasive squamous epithelial cancer of the cervix has died of her disease, aged 32. Diagnoses of malignancy other than cervical neoplasm have been noted in 16 women: cancer mammae in 5, cancer ovarii in 2, adenocarcinoma corporis uteri 2, cancer pancreatis 2, cancer ventriculi 2, cancer renis 1, cancer recti 1, and skeletal tumor UNS in 1 case. Of these, 11 have succumbed to their disease. A further 10 women have died of other, non-malignant disease. The various diagnoses of malignancy (other than cervical cancer) stand in relation to each other as anticipated. However, the material is too small to make an

exact assessment of any increase in the incidence of cancer in these women, apart from that of cervical cancer.

Those women who were married at the time of their spouse's gonorrhea (Groups i and ii) had an average age at the time of the evaluation of 57 years (range 41-77). Those women who were unmarried at the time of their future spouse's gonorrheal infection (Group iii) were, on average, younger: 53.2 years (range 36-70). The average age of the former at the time of diagnosis of cervical malignancy was 44 (range 29-74), and of the latter, 39.8 years (range 25-55).

The incidence of cervical neoplasm in wives of men who had been married 2-5 times has been compared with that in women who had been married only once, but no statistical significant difference was found, either in the women who themselves had had a gonorrheal infection or in the others ($\chi^2 = 0.07$ vs. 0.28).

Only in one case had repeated cervical neoplasm occurred among wives of men married several times. The first and the third wife of a three times married man had contracted CIS. Neither of these 2 women had herself had a gonorrheal infection. One other pair of women has previously been known where the one was the man's infection source and who later contracted CIS, while the other, the man's wife, contracted CIS 12 years later. Only the latter woman was included in the present material.

The wives of those men who had been married several times have not shown a higher frequency of malignancy. Nor had cervical neoplasm occurred more often in women who had previously been married with another man.

DISCUSSION

In a previous investigation it was found that invasive and preinvasive cancer of the cervix had occurred in 22.6% of those women who had had a gonorrheal infection 24-23 years earlier. The purpose of the present work was not to discuss what agent(s) may be of importance in the develop-

ment of cervical neoplasm, but to investigate whether there is an increased frequency of such among women following a gonorrheal infection in their sexual partner. If this were to prove true, it might mean that a possibly carcinogenic agent is transferred from the man to the woman to a greater extent together with a gonorrheal infection and that the agent remains with the man even after he has undergone treatment for the gonorrhea.

One difficulty with the material collected was the long lapse of time that had to be surveyed. It was necessary to allow a sufficiently long latency period so that any malignancy in the woman might have shown itself (5). It was decided that a period of at least 10 years must have elapsed since marriage. This is perhaps not sufficiently long, but since it can be assumed that sexual relations between future spouses had taken place even before marriage, this period can be considered even longer.

In the present study, the age-matched population of Malmö municipality served as a control. Considering the high participation in the cytological screening (92%), such a control group is the best one available for a retrospective epidemiological study of the frequency of the cervical malignancy. It would be desirable to match the controls even according to their sexual anamneses, including their male partners. However, we found it impossible to perform such matching without encroaching on the personal integrity of the patients.

It was a surprise that as many as 23 wives had escaped infection even though their husbands had had a gonorrheal infection. Lengthy absence from one's wife, illness, pregnancy and sexual disinterest are the usual reasons given for the freedom from infection.

The loss of 63 of those women comprising the original material can be thought large, but allowance must be made for the long period covered by the investigation, in which case the drop-out rate can be considered reasonable. Of 63 women, nothing was known of the cervical status in 20 and

15 had had some cytological change such as mild dysplasia or, most often, moderate dysplasia. Having regard to the fact that these women fall within a risk group, they were invited to an extra gynecological screening, but failed to appear. It is to be feared that one or several will one day have an invasive cervical carcinoma. Women who had separated from the man in question before his gonorrheal infection should not run any greater risk than the average woman. On the other hand, nothing is known about those women who were excluded merely because they had been married less than 10 years. An attempt was made to correlate the risk to the time elapsed from the woman's/husband's gonorrheal infection until the diagnosis of the cervical neoplasm. The spread in time has been particularly long, from 5 up to as long as 35 years, yet without any apparent pattern either within or between the groups.

Women who themselves had had a gonorrheal infection were found to have had a cervical malignancy in an even greater frequency than in a previous investigation. The explanation may lie in the longer observation period and thus the women's higher average age. A statistical difference vis-à-vis the control group exists for those women who have never had a gonorrheal infection and most probably had not met their future husband at the time of his gonorrheal infection. The same applies to women who were married at the time of their husband's infection but who themselves did not become infected. Here, the contact was with all probability more intimate shortly after healing of the man's gonorrhea.

Apart from those women who themselves have had gonorrhea, it can nevertheless be stated that it appears to be the man's infection that is the common factor in women who have a greater frequency of cervical neoplasm. On the basis of this material, however, one cannot say that the occurrence of cervical neoplasm has been more common among women who have been married several times than among those married only once. One might assume that the risk of

coming into contact with a certain agent increases with the number of men a woman marries, but if her first husband is the bearer of that agent, then it is of no consequence if the woman remarries several times.

If one assumes that the man is the bearer of an agent which is transferred to the woman, it seems remarkable that not all the women he has been married with have become infected, particularly those married with a man whose former wife has developed a cervical malignancy. First wives are not affected more often than second or subsequent wives. The only explanation for this seems to be that there are individual differences among women in susceptibility to an agent, or a certain predisposition to develop cervical malignancy. Only once has CIS been observed in two wives of a three times married man, and the woman who had a normal cervical status was the second of the three. The first wife had been married with the man at the time of his gonorrheal infection, yet not become infected. Four years later, at the age of 29, she was operated on for CIS. The third wife was operated on for CIS at the age of 42, 23 years after her husband's gonorrheal infection. In another case, the one woman, who actually infected the man, was excluded from the material, since they were not married and she was merely the infection source. This woman had CIS diagnosed 11 years later at the age of 50, and the wife of this man was similarly diagnosed 12 years later at the age of 45.

A lower average age in women who have not been married at the time of their future husband's gonorrhea renders it probable that further cervical malignancy in this group will make its appearance. A younger average age at the diagnosis of cervical malignancy in women unmarried at the time of their future husband's gonorrhea, than in married women, may be an expression of the above, though yet another explanation is conceivable: married women have come in contact with the agent at a higher age than those women who were unmarried at the time of their future

husband's gonorrhea. When marrying, they were as a rule younger. If the interval from contact with the agent until diagnosis of cervical malignancy is on average equally long for married as for subsequently married women, the younger women will contract cervical malignancy earlier. Should this be so, it can be taken as further evidence of the connection between the man and his wife's cervical malignancy, since it implies the decisiveness of the time of the first contact with the man in question and the time when the cervical malignancy arises.

In this epidemiological study an attempt has been made by using a not too uncommon sexually transmitted disease, gonorrhea, to trace another disease whose epidemiological character is still insufficiently elucidated. We have used simple contact studies and come to the conclusion that earlier suspicions about the possible sexual transmission of a carcinogenic agent from man to woman were well founded. It has also been found that it is evidently not all women who contract cervical malignancy even though they might have been exposed to a carcinogenic agent.

The next step should be to ascertain if there is any difference between women who can be considered exposed to the same carcinogenic agent yet who react differently by developing or not developing cervical neoplasm. Even families where several members have had cervical malignancy might well be suitable in the search for genetic indicators in the form of defective immunological defence (Stig Kullander, personal communication). Immunological defects can often be correlated to some agent(s) that can be connected with the cervical malignancy. This is perhaps a more practicable approach than is searching after a specific carcinogen among several possible such agents in an already developed cervical neoplasm.

ACKNOWLEDGEMENT

I am grateful to Jonas Ranstam, Institute of Statistics, University of Lund, for valuable help with the statistical analyses.

Table I. Occurrence of preinvasive and invasive cancer colli uteri in women whose husbands had had a gonorrheal infection 26-37 years previously (n=232)

Group	Women with CIS or invasive ca colli uteri	Women found healthy at latest gynecological investigation, including vaginal smears	Total
i Man married at time of his gonorrheal infection; wife infected	17* + 2** (27.1)	51	70
ii Man married at time of his gonorrheal infection; wife not exposed to infection/ negative gonorrheal culture	5* (21.7)	18	23
iii Man unmarried at time of his gonorrheal infection; women without anamnestic record of gonorrhea	19* (13.7)	120	139

*CIS; ** invasive ca colli uteri.

Figures within parentheses are percentages.

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FAMILIAL OCCURRENCE OF CERVICAL CANCER, STAGES 0 - IV

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Abstract. All patients hospitalized in 1982 at the Department of Gynecology in Malmö because of malignancy of the cervix uteri attended an interviewing study concerning the presence of cervical cancer among their nearest relatives. Besides, earlier gonorrheal infection was asked for. The blood group was determined as was the secretory status and Gm allotyp. As a control group the family of the male consort was used.

Cervical cancer was found significantly more often in the patient's mothers (7.9%) than in the consort's mothers (1.0%). Sisters, aged 20 or over, of the patients had cervical cancer significantly more often (7.5%) than sisters of the consorts (1.1%). All over, cervical cancer in mothers and/or sisters was found in 15.6% of the patients. In case of invasive cancer or previously operated CIS this figure was 17.5%.

The patients did not differ significantly from the normal population regarding blood group and secretory status. A somewhat lower, although non-significant, frequency of Gm(1) allotyp in patients with invasive cancer compared to patients with CIS was found. Patients with a positive family history for cancer had more often had gonorrhea (24.0%) than patients with a negative family history (18%).

The study indicates a multifactorial etiology for cervical cancer.

INTRODUCTION

In man, most forms of cancer have as a rule only a slight familial tendency. However, a greater familial risk has been demonstrated especially in those suffering from mammary or colon cancer. A familial accumulation of this sort offers prospects of early detection and treatment of high-risk individuals. Charted accumulations of such familial clustering can also be used in etiological cancer studies. Apart from such accumulations, genetic predisposition can be suspected in patients whose cancer debut occurs at an early age or who are affected by a multiple cancer form. Not until we have suitable biological indicators at our disposal can this lead to improved diagnosis of risk cases.

Only an occasional report in the literature has pointed to a suspected familial, possibly genetic disposition in cervical cancer. There have been cases of families in which several members have been affected by cervical cancer. Three such families in which both mother and a daughter had cervical cancer are described (1). Earlier had been reported stage 0 cervical cancer in 3 sisters (2,11). A "respectable family with conservative catholic habits and stable marriage, with all suspicion of promiscuity completed excluded" in which 3 daughters out of 7 had been affected by preinvasive cervical cancer had been reported (4). No prospective hereditary studies of cervical cancer have ever been published and the likelihood of a "first-degree" relative of a woman with cervical cancer likewise having or getting the same complaint is unknown. The genetic mechanisms which could be responsible for such a familial predisposition have not been studied.

We have therefore made a systematic prospective investigation into the occurrence of the disease in close relatives of all patients with cervical cancer who were hospitalized and treated at the Department of Gynecology, Malmö, in 1982. The decision to conduct this study was

prompted by our own earlier observation of several families having a remarkable accumulation of cervical cancer.

If such circumstances as low age at first coitus and first pregnancy, numerous sexual partners, low socio-economic class, and genital infections, whether specific or not, were relevant to the occurrence of cervical cancer, it would not be surprising if lifestyle models and social environment were to lead to cervical cancer in mothers and sisters. Whether this familial factor exercises its influence by environmental pressure or by genes is a contentious matter, but is more likely to result from a combination of these. Thus individuals with a high cancer risk can have been born with a genetic predisposition and subsequently been exposed to one or more carcinogens. Another factor, gonorrhea (5), is connected either directly or indirectly with a greatly increased cancer risk 10-15 years after the infection, since more than 20% then have been affected by cervical cancer, either invasive or non-invasive. In our prospective study - carried out on the same Malmö population as that in our earlier study (5) - we therefore attempted to chart, in addition to familial accumulation, previously experienced gonorrheal infections.

Probably there is a connection between blood group or other genetic markers and certain cancer forms as well as certain infections. A significant overrepresentation of blood group A in cases of invasive cervical cancer had been demonstrated (9). No study appears to have been made of non-invasive cervical cancer. There have been reports of increased sensitivity to gonorrheal infection among individuals belonging to blood groups E and AB (8) which has been attributed to the absence of anti-B isohemagglutinin. The idea has even been put forward that non-secretory properties for blood group antigen might be involved in host-agent interaction, thus further increasing the sensitivity to bacteria in persons having blood group B (8). We therefore determined both blood group and secretor

status in all patients in the investigation. The frequent presence of gonorrhea and other vaginal infections concomitant with cervical cancer ought to indicate that these women suffer from immunological deficiency and a weak IgG-antibody response to the antigen of the infecting organism. Several investigations have indicated a connection between Gm(1) allotype and immune response (6), and between Gm allotype and the ability to overcome infectious diseases. In the hope of discovering a biological marker for cervical cancer susceptibility, we therefore determined also the distribution of Gm(1) individuals among the investigative material.

MATERIAL AND METHODS

The patient group studied comprised all those with a histologically proven CIS or invasive cervical cancer who were hospitalized and treated at the Department of Gynecology, Malmö, in 1982. The patients were interviewed whilst in hospital, in connection with the scrutiny of their completed standardized family anamnesis, distributed some days previously. Ample time was allowed for contacts and questioning between the patient and her family. All patients agreed to cooperate and only one, who suffered from schizophrenia, was unable to collaborate. Information regarding malignant diseases was collected regarding all "first-degree" relatives (parents, siblings, children) of the patient and her consort. The interview regarding diseases among the family of the consort was carried out by using the same questionnaire. All reports of cervical cancer in these relatives were checked by inspecting their medical journals, histopathological reports, or obduction reports. Those patients interviewed were, in the main, well informed about their relatives' medical histories. However, our study covered only "first-degree" relatives, since information on more distant relatives seemed to us less reliable. All the patients were questioned further about any previous bout of gonorrhea and when it occurred.

They were also requested to give samples of blood and saliva for determination of blood group, Gm-typing and secretor status. Blood group antigen was determined by standard methods. Secretor status was determined by testing a saliva sample by boiling for 20 min, followed by centrifugation at 500 g for 10 min. Thereafter the supernatant was kept at 4°C until it was time for the blood group antigen assay.

The control data used for blood group frequency in Sweden are based on studies on 137,762 subjects (10). The control data for secretory status are based on tests made on plasma samples taken from blood donors living in the same geographical area (7). Gm-typing was carried out according to standard methods (6). The gene frequency of Gm(1) in a Swedish population is based on an analysis of 2,917 individuals. Statistical analyses carried out with standard methods.

The composition of the material is shown in Table I. The majority comprised cases of definite cancer in situ (CIS) numbering 128, 12 had histologically atypia. Twenty-one women comprised a special group, having been treated previously at the clinic for the same complaint. Nineteen women, the oldest, had invasive cervical cancer.

RESULTS

Fourteen of 177 mothers (7.9%) of our present patients had had cervical cancer, as had 14 of 188 sisters aged 20 or over, i.e. 7.5% (Tables II, III). By contrast, only one of 103 mothers (1.0%) of the known male consorts had had the disease, and only one of their 93 sisters (1.1%) in the same age. The differences are statistically significant ($p < 0.01$). Altogether 28 patients (15.6%) among the 180 had at least one first-degree relative with cervical cancer (Table IV). Three of the 180 had both mother and a sister with cervical cancer. Even when only patients with a consort anamnesis are compared with the consort's family, the same statistically significant difference is found.

The smaller number of known mothers and sisters of consorts than of patients is a consequence of death or divorce or lack of contact, which made it impossible to trace the consort's family situation.

The patients themselves had altogether 37 daughters aged 20 or over. However, none of these was reported to have received treatment for cervical cancer.

Familial cervical cancer was more common when the patients' affection was of a more serious nature. Thus the frequency was 13.6% (5 out of 37 mothers) for invasive cancer or CIS cases requiring repeat therapy, vis-à-vis 6.4% (9/140) in CIS and atypia cases.

There had been 34 cases (18.9%) of gonorrhea anamnestically in the whole material (Table V), but 6 cases (24.0%) among women with familial cervical cancer. This indicates a multifactorial etiology for cervical cancer with familial clustering. A weakened immune defence could conceivably be a hereditary etiological factor triggered off by a particular lifestyle or environmental factors.

It did not prove possible in the material as a whole to demonstrate any significantly altered blood group distribution or non-secretory characteristic, or absence of Gm(1) allotypes, when comparison was made with the normal population, nor in those previously affected by gonorrhea. Cases of cervical cancer in the familial anamnesis admittedly showed a different and particular blood group distribution, with a preponderance of blood group A (Table VI) consistent with that reported earlier for invasive cervical cancer (9), but our material was too small for conclusion about validity.

Gm(1) was more often lacking also in the most serious forms - invasive or reoperated CIS, compared with simple CIS (Table VII), though the difference is not statistically significant.

DISCUSSION

In altogether 26% of the whole patient population there was either an anamnestic report of previous gonorrheal infection or of cervical cancer in a near relative. This is sufficiently significant as a phenotypic indicator to warrant careful scrutiny of such patients coming for gynecological check-ups and in such cases conduct more genetic, familial and immunological investigations with a view to establishing the genetics and prognosis of cervical cancer.

Great difficulty has been encountered in obtaining a control material that is clinically suited to studies on cervical cancer. An ideal material would be women from the same geographical area, matched for age, parity, age at first coitus and first pregnancy, same contraceptive mode, socio-economic station, and numerous other variables. Instead of such an analysis, however, we chose as our control material for comparison, cervical cancer frequency in near relatives to the consort. These control subjects probably bear a greater similarity to the patients' relatives as regards lifestyles, socio-economic status and occupation than does the general population.

Investigations into genetic factors in cancer have previously been hindered by difficulties in objectively establishing the cancer type in relatives, due to tenuous family relations and inadequate disease diagnostics. These difficulties are nowadays more easily resolved and were of little consequence in the present investigation. In the near future it ought to be possible even to study mothers as hereditary carriers, as distinct from fathers (affected maternal and paternal sisters).

Those cases of familial accumulation of cervical cancer that we have observed could obviously be due to common environmental factors; i.e. the milieu has been "inherited". The differences in the frequency of gonorrhea indicate this and it is always difficult to exclude a subpopulation with a heterogeneous way of life. Probably

are, however, other pure genetical factors, at least partly, involved. Studies of all or more patients with cancer in a homogeneous population - as in Malmö - offer advantages methodologically because the risk of bias will be decreased.

A more general familial predisposition to malignancy cannot be excluded in our patients. Certain organs or patterns of organs might be affected among relatives. We will later on work up our data from this point of view.

A tendency to early and bad prognosis has been described in case of familial occurrence of cancer. Those of our patients whose mothers were affected had more often clinically advanced tumours.

The taking and evaluation of the medical history of near relatives as regards to cervical cancer, and of course other cancer forms, is often defective although potentially the technique is simple and cheap in order to shed light on the etiology of cancer and, furthermore, the method might be helpful in the prevention of cancer. The practical consequences for patients with a high familial risk are inadequately penetrated.

Previous bouts of gonorrheal infection should be added to the risk exposure of patients who are candidates for surveillance and prophylaxis. Risk patients who are deemed suitable for active prophylaxis should as a rule have completed their families and be over 35 years of age. For risk patients of this age who undergo laparotomy for other reasons and for whom subtotal hysterectomy is considered, total hysterectomy should be recommended instead, since the accumulated morbidity arising is of minor proportions.

Table I

Composition of the investigation material

	n	With consort anamnesis n	Without consort anamnesis n	Average age (range)
Cervical atypia	12			
CIS	128	87	53	36.4 [±] 9.8 (21-67)
Previously ope- rated CIS	21	11	10	34.6 [±] 10.0* (22-61)
Invasive cervical cancer, stage IB - IV	19	7	12	56.4 [±] 15.7 (28-84)
Totals	180	105	75	

*At first CIS diagnosis 29.9[±]9.8 (21-55)

Table II

Cervical cancer in the mothers

	<u>Cervical cancer in mothers</u> <u>of patients</u> <u>of consorts</u>	
Cervical atypia and CIS	9/140	1/85
Previously ope- rated CIS	3/21	0/11
Invasive cervical cancer stages IB - IV	2/16	0/7
Totals	14 ^a /177 ^b (7.9%)	1/103 ^c (1.0%)

a Seven of these were mothers of patients with consort anamnesis

b Three further cases lacked relevant information

c Two further cases lacked relevant information

Table III

Cervical cancer in sisters

	<u>Cervical cancer in sisters ($\geq$ 20 years) of patients</u>		<u>of consorts</u>
Cervical atypia and CIS	12 ^a /141		1/75
Previously ope- rated CIS	1/20		0/5
Invasive cervical cancer stages IB - IV	1/27		0/13
Totals	14 ^b /188 (7.5%)		1/93 (1.1%)

a Of which 2 cases were in sisters to one patient

b 10/103 cases when only sisters to patients with consort are recorded

Table IV
 Familial cervical cancer in mothers, mothers and sisters, or
 sisters, of patients

	n	Mothers only	Mothers and sisters	Sisters only	Totals
Cervical atypia and CIS	140	6	3	9	21/140 (15.0%)
Previously ope- rated CIS	21	3		1	4/21 (19.0%)
Invasive cervical cancer stages IB - IV	19	2		1	3/19 (15.8%)
Totals	180	11	3	11	28/180 (15.6%)

Table V

Bouts of gonorrhea anamnestically

	n	Anamnestic gonorrhea	Average age when affected (range)
Cervical atypia and CIS	140	23 (16.4%)	22.0 ⁺ -6.4 (14-40)
Previously ope- rated CIS	21	6 (28.6%)	22.3 ⁺ -6.5 (16-29)
Invasive cervical cancer stages IB-IV	19	5 (26.3%)	22.0 ⁺ -2.1 (20-25)
Totals	180	34 (18.9%)	

Table VI
Blood grouping of cervical cancer patients with/without close relatives
with the same disease
(n = 180)

	A	B	AB	O
Cervical cancer in close relative	14 (56.0%)	2 (8.0%)	1 (4.0%)	8 (32.0%)
No cervical cancer in close relative	64 (41.3%)	19 (12.3%)	6 (3.9%)	66 (42.5%)
Normal population	46%	10%	4%	40%

Table VII Presence of Gm(1) in the whole material compared with the normal population

	Positive	Negative	Not investigated
Cervical atypia and CIS	83 (62%)	51 (38%)	6
Previously ope- rated CIS	9 (45%)	11 (55%)	1
Invasive cervical cancer stages IB-IV	7 (50%)	7 (50%)	5
Totals	99 (59%)	69 (41%)	12
Normal population	58%	42%	

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XENOTRANSPLANTATION OF HUMAN CERVIX UTERI INTO NUDE MICE
Histological Studies of Transplants with Normal Tissue and
Preinvasive and Invasive Squamous Carcinoma

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Abstract. Biopsies of human portio was transplanted subcutaneously into nude mice. Three types of tissue were used: preinvasive and invasive squamous cell carcinoma and normal tissue. Some animals were dosed parenterally with estrogen or progesterone. Microscopically the transplants revealed degenerative changes in stroma and epithelium. Vital squamous epithelium was nevertheless observed up to the 10th postoperative week (normal epithelium), to the 8th week (preinvasive cancer) and to the 34th week (invasive cancer). In normal and preinvasive cancer epithelium, mitoses occurred until the 4th week. The stroma became hyalinized in some transplants, while in others it was invaded by murine vessels and after a time became merged with the mouse tissues. There were no differences in the appearance of transplants from estrogen-treated, progesterone-treated and untreated mice. The method is hampered by the degenerative changes in the transplant. In consequence it is difficult to evaluate the effects of medication or other manipulations.

INTRODUCTION

Since the first xenotransplantation of human tissue into nude mice (1), attempts have been made to transplant most types of human tissue into these laboratory animals. Transplanted neoplastic human tissue can serve as a model for cell kinetic studies and dosing of the recipient animals with various pharmaceuticals makes it possible to study their effects on the implanted tissue. Administration of cytostatics or hormones to tumour-bearing mice constitutes one model for testing the effects of therapy on the individual tumour, or to study the effects of external irradiation (2).

Xenotransplantation of premenopausal human portio has been performed by Eichholtz et al. (3). Histological examination showed preserved squamous columnar epithelium in 12 of 24 transplants. The transformation zone between the squamous epithelium and the columnar epithelium is of importance in early carcinogenesis and an established tissue implant could be an invaluable complement to experimental studies of carcinogenesis. Tissue of pre-invasive cancer colli uteri could, when implanted in nude mice, facilitate the study of transition to invasive cancer. Teufel et al. (4) found continuous growth in 50% of transplanted invasive cervical tumours. Sharkey et al. (5) did not succeed in attaining growth of primary epidermoid carcinoma of the cervix but observed growth in 3 out of 4 cases of metastatic tumour. Huck et al. (6) succeeded in transplanting cutaneous metastasis with continuous growth and tested cytostatics and combinations thereof.

Estrogen can experimentally increase the incidence of cervical cancer in certain mouse strains, whereas other animal species show no such increase. It has not been demonstrated that progesterone increases the frequency of cancer in animals (7).

In a small series of nude mice, we have studied the histological characteristics of normal human portio

tissue, and of preinvasive and invasive squamous cell carcinoma following implantation of biopsy material. In order to study possible hormonal effects on the implanted tissue, a number of the mice were treated parenterally with estrogen and others with progesterone.

MATERIALS AND METHODS

To carry out xenotransplantation of normal portio tissue and of tissue affected by preinvasive or invasive squamous cell carcinoma, we used 5-6-week-old female nu/nu nude mice (obtained from Gl. Bomholtgaard, Denmark). Normal portio tissue had been taken from women of fertile age in connection with uterine abrasion. Preinvasive cancerous tissue was localized colposcopically and removed prior to conization. Cervical tissue affected by invasive squamous carcinoma was removed by knife from an area with the least necrotic tissue. Until implantation was effected, within about 15-20 minutes, the tissue samples were kept in medium 199. Four tissue pieces were transplanted into each of 3 mice, one piece in both front and hind region on either side. We attempted to obtain tissue pieces 3 mm in size. Peripheral tissue from the biopsy area was fixed in formalin for subsequent comparison.

In some cases the animals were given estrogen or progesterone (Table I).

Table I. Distribution of material as regards tissue origin

	Total no. of cases	Those treated with hormones
Normal portio	8	4
Preinvasive carcinoma	14	6
Invasive carcinoma	16	8

Control transplants not intended for hormone administration were always taken from the same patient. Hormone-

-treated mice received 5 mg Depo-Provera (Upjohn) intramuscularly every 14 days, or 0.02 mg Estradurin (Leo) subcutaneously each week. The pharmacological effects were checked at the time of sacrifice. Mouse vaginal epithelium evidenced a noticeable difference between the estrogen- and progesterone-treated groups. The former had a cornified squamous epithelium, while the latter had a mucinous columnar epithelium.

With the intention of obtaining a longer series, some of the specimens were transferred to fresh animals when the original mice became cachectic. However, no systematic transfer was made.

In most cases a specimen was taken each week for histological investigation but this was not done consistently as we wished to study some of them at a later time and healthy mice were therefore allowed to survive longer with their transplants. Moreover, some mice were sacrificed when in poor condition and all transplants removed at the same time. Some transplants were also recovered from animals that died spontaneously. In certain cases, some transplants were used for investigations other than microscopy. Consequently the numbers of transplants varied on the different investigation occasions. It would therefore be misleading to report the numbers of positive transplants as a measure of the success of the experiment at any one timepoint. For this reason we have regarded as a positive result at least one successful transplant from any one animal at any one investigation timepoint.

After removal from the host, the transplant was fixed in 10% formalin and subsequently divided into two portions which were embedded in paraffin wax. Serial sections were taken at 4 or 5 levels, depending on the size of the sample. The sections were stained with hematoxylin-eosin. Peripheral biopsies from the portio were treated in the same way.

The composition of the material is shown in Table I. As mentioned, the number of transplants varied from case to

case, but the total number in all investigation series amounted to 262.

The following factors were assessed microscopically: relationship between transplant and surrounding murine tissue, appearance of stroma and vessels in the transplant, presence of vital columnar and squamous epithelium and of vital cancer. Squamous epithelium was assessed as normal, atrophic or degenerating; or as dysplastic, or with preinvasive or invasive cancer.

RESULTS

Relationship of transplant to mouse tissue

Actual inflammatory changes were seldom observed in mouse tissue. In one case of preinvasive cancer, there was abscessing around the transplant, which was badly decayed. Patient biopsies from the transitional zone were rich in granulocytes in the cervical crypts, which may denote bacterial infection. Otherwise there was no granulocyte reaction in either normal cases or in cases of preinvasive cancer. In transplants containing invasive cancer there was often widespread necrosis and granulocytic infiltration was found around 3 of 8 such transplants.

Where squamous epithelium from the transplant touched murine tissue, histiocytic reaction was invariably observed, with formation of a structure resembling granulation tissue and having foam cells and occasionally multinucleated giant cells adjoining keratinized cell lamellae from the transplant (Fig. 1). In this tissue there was no noticeable capillary proliferation. This reaction in the host animal was the same, whether the implanted tissue originated from normal epithelium or from cases of preinvasive or invasive cancer, and there was no difference in hormone-treated animals.

Some transplants had been removed almost completely free from adhering host tissue, which would indicate a low-grade coherence. Most cases, however, showed varying

amounts of adhering mouse tissue, subcutaneous fat or muscle. In these cases the border between the transplant and mouse tissue was usually indistinct being bridged by small vessels and connective tissue cells. In most transplants small aggregates of blood pigment were seen in the border region and in many cases there was also an increase in mast cells in the surrounding murine tissue.

Transplant stroma

Stroma in the various transplant groups showed a uniform picture and the changes evidenced no consistent relationship to hormone dosage. All transplants showed some form of degenerative change in the stroma; three were completely necrotic. The most remarkable change otherwise was a reduction of or decomposition of the interstitial tissue in the transplant, which was often manifestly developed after 1 week. The vessels in the transplant were closer together, and capillaries and veins were wider than in normal portio stroma. They often showed a loss of the littoral endothelial cells, while the vessel lumen was criss-crossed by a network of endothelial cells (Fig. 2).

In most cases fibrin thrombi were found in occasional vessels. The arterial walls were partially hyalinized. In the areas of decomposed stroma, a sparse infiltration of granulocytes was evident even during the first week and also, to a varying degree, histiocytic foam cells. Minor calcifications were often seen, but no major ones.

The decomposing areas of stroma were invaded to a varying extent by vessels from the host. They often formed a peripheral rim of thin-walled newly formed vessels (Fig. 2) but also penetrated deep into the transplant along vessels and septa. Ten specimens from 7 cases (2 normal, 4 preinvasive and one invasive cancer) had large areas of strongly hypercellular stroma, probably proliferation of murine fibroblasts from the invading granulation tissue. Changes of this nature were found in transplants from 1 week old up to the 20th week. During the first 4 weeks

there was scarcely any systematic difference in the appearance of the stroma. Subsequently there was a tendency to a more advanced hyalinosis, but there were too few cases in the study, which had been in progress for quite some time, for any reliable conclusions to be drawn regarding the temporal progress of these stromal changes. In the oldest transplants there was often a rim of compact collagen in the periphery apart from a generally advanced hyalinosis, but there were also cases that lacked the original stroma almost completely and where the remaining epithelial structures were surrounded by murine subcutaneous tissue. This was true not only of invasive cancer but also of epithelial formations of benign type. Cervical glands could be observed with preserved basement membranes which were entirely surrounded by murine subcutaneous tissue without any remaining portio stroma.

In five investigations, no transplant was found. Instead, a mouse lymph node was found (these five specimens derived from 4 cases - 2 of preinvasive cancer, 2 of invasive cancer). Transplantation had taken place between 4 and 16 weeks earlier. One mouse was treated with estrogen, another with progesterone, and the remainder had not been subjected to hormones. In one of these lymph nodes, small necrotic remnants of transplant were found, surrounded by multinucleate giant cells in the centre of the node. Another of the lymph nodes contained in its centre a large aggregation of foam cells, probably remnants of resorbed transplant tissue.

Epithelium in the transplant

A. Cases with normal epithelium: The series of 8 normal cases displayed normal squamous epithelium (SE) on the surface in peripheral biopsies, without exception. Seven of the cases showed preserved SE in at least one transplant. The epithelium was depressed into a bowl-like form (Fig. 1) which could generally be demonstrated to be connected with the surface of the transplant, though in

some cases the epithelium took the form of a cyst in the stroma without demonstrable connection with the surface. The amount of preserved SE varied appreciably. In some transplants it formed no more than a little island, while in other cases the entire bowl or cyst was filled with preserved SE. This variation lacked any discernible pattern and was not correlated with the duration of implantation or with hormonal treatment. Without exception the SE displayed some divergence from the normal, but in some transplants these differences were small and took the form of occasional swollen and vacuolized epithelial cells and dispersed apoptotic bodies present at various levels. Similar relatively well preserved SE was found on occasion in 3 cases, but never after 3 weeks of implantation. The other cases with preserved SE displayed more prominent abnormalities, either in the form of pronounced atrophy, where the epithelium consisted of no more than 2-3 flattened cell layers, or of more obvious degenerative features, with large swollen cells having a pyknotic nucleus and completely irregular keratinization, generally a combination of both types of changes within different parts (Fig. 3). Many transplants had a focally or completely necrotic SE, where even the basement membrane was destroyed. The bowl was often filled with keratinized or parakeratotic cells.

In one transplant, mitoses were observed in the SE even in the 4th week, but never subsequently. One case had a degenerated but identifiable SE even after 10 weeks in an estrogen-treated mouse. Progesterone-treated mice displayed vital SE for as long as 5 weeks, control mice for up to 8 weeks, but the series are small, and discontinuous in the later weeks, and no conclusions can be drawn from these figures. There was not a single case with a series of preserved SE for more than 3 consecutive weeks and during this period no change in the epithelial picture could be observed.

Columnar epithelium (CE) in cervical crypts was found to be preserved in 6 of the 8 cases in at least one specimen. It seemed to be more resistant to the experimental conditions, was more abundant and often covered the crypts completely. In one case, preserved CE was observed after 10 weeks. However, the cells displayed variable abnormality, often in the form of a reduced mucus formation or of pyknotic nucleus. Within one and the same transplant, CE could look quite normal in places, while in other parts it was severely atrophic or degenerated.

No consistent morphological differences could be observed in any respect between the estrogen- and progesterone-treated transplants, in comparison with the control specimens.

B. Epithelium in preinvasive cancer and dysplasia: In this series of altogether 14 cases, preinvasive cancer or severe dysplasia was found in only 9 instances in peripheral biopsies. Yet the diagnosis preinvasive cancer had been confirmed in the surgical examinations in every case. A positive transplant was retrieved on one occasion in a case that lacked malignant changes in the peripheral biopsies, which illustrates the unpredictable nature of the method. Three cases of hormone administration proved positive for CIS or dysplasia in at least one transplant, but there was no difference in appearance between estrogen-progesterone- and control specimens. These cases are therefore described together with others below. Of 14 cases, only 4 showed at least one transplant with a definite picture of preinvasive cancer. Three of them had on another occasion a transplant displaying dysplasia. Only a single case showed preinvasive cancer on more than one occasion. Altogether 7 transplants in the series proved positive for preinvasive cancer - one after 1 week, two after 2 weeks, three after 4 weeks and one after 8 weeks.

Dysplasia without a clear picture of preinvasive cancer was more common. No grading of the dysplasia has been made, as the degenerative epithelial changes which occur even in normal cases make demarcation between dysplasia groups a hazardous matter. Eight cases showed on some occasion at least one transplant with dysplasia (altogether 17 positive transplants). Even here a maximum of 8 weeks was reached. No change in the picture could be observed in cases with a positive result on more than one occasion (one case had 6 positive transplants during 4 weeks). Altogether 9 cases had preinvasive cancer and/or dysplasia in at least one transplant in some investigation. In the hormone groups there were altogether 5 mice with a positive transplant following estrogen treatment, the longest surviving to 8 weeks. Four progesterone-treated mice had a positive transplant, the longest surviving to 4 weeks.

A number of mice had transplants with vital epithelium of a non-malignant nature. Altogether 26 transplants had preserved SE without dysplasia and altogether 60 had preserved CE with varying grades of abnormality corresponding to the normal group. Vital CE persisted at most to 13 weeks in control mice, degeneratively changed SE until 9 weeks in progesterone-treated mice; otherwise the survival times in the normal group were not exceeded.

The histological picture of epithelium in transplants with vital dysplastic and cancerous epithelium was in principle the same as in normal cases. There were varying amounts of epithelium, but often no more than one tiny island in a crypt. As in normal cases, the atypical epithelium seemed to be better preserved in the crypts than on the transplant surface. Also the dysplastic epithelium showed varying grades of degenerative phenomena, with swollen cells, nuclear pyknosis, etc. Mitoses, even atypical ones, were observed up to the 4th week (Fig. 4). Infiltration in mouse tissue never occurred. One case had a peculiar appearance after 4 weeks (Fig. 5). The

stroma of the transplant was merged with murine connective tissue. There was a well preserved epithelium, both CE and more or less dysplastic SE and even frank preinvasive cancer. Because of the merging of stroma with mouse tissue, the transplant had been removed together with mouse skin. Broad contact had become established between the transplant's dysplastic SE and SE in the mouse skin, both basal epidermis and follicular epithelium. The epithelium of mouse and transplant merged completely. There were intercellular bridges between the transplant's squamous epithelial cells and cells containing coarse keratohyalin granules which indicated their murine origin.

C. Transplants with invasive cancer: In 6 of 16 cases there was no cancer in the peripheral biopsies, but the tumour could be studied in surgical specimens. On some occasion a positive transplant was retrieved in 4 of the 6 cases. Ten cases thus had positive peripheral biopsies. In 6 of them no positive transplant was ever retrieved. Eight cases out of 16 showed on some occasion at least one transplant positive for vital cancer. In the non-hormone group at least one positive transplant was retrieved in 7 of 16 cases, in the estrogen group in 4 of 7 cases, in the progesterone group in 5 of 8 cases. In the non-hormone group there were mice with a positive transplant up to the 8th week, while estrogen-treated mice were positive at most to the 20th week and one progesterone-treated mouse had a positive transplant for as long as 34 weeks. It should be noted, however, that only 3 cases were positive after the 8th week, one only in a progesterone-treated mouse, one only in an estrogen-treated mouse and one in mice treated with both. No obvious differences in transplant survival could be discerned in the different groups. The case with most positive transplants also had most positive "takes" in both estrogen- and progesterone-treated mice and was moreover the case that had the longest transplant survival of all. The finding in this

individual case can be taken to mean that it is the aggressiveness of the cancer itself that determines the frequency of "take" and transplant survival, rather than any substance supplied.

The histological picture in the positive transplants varied considerably. In some cases there was only an occasional vital cell island within an otherwise hyalinized or necrotic transplant, while on other occasions there was much vital tumour tissue with only minor remnants of stroma (Fig. 6).

It is impossible to draw any conclusions from these findings, since one cannot know the original composition of the transplant as regards the bulk of vital cancer tissue in relation to necrotic tumour or stroma. It is exceedingly difficult to express an opinion on whether the tumour infiltrated beyond the transplant's original limits, as the latter can evidently be replaced by mouse tissue. Thus a mixture of cancer and murine tissue can just as easily be cancer infiltrated by mouse tissue, as vice versa. However, 2 cases showed a convincing picture of infiltration outside the original limits of the transplant. These were 2 cases with the longest survival in the whole series, which indicates that the tumour in both instances was unusually aggressive. However, those transplants were not the longest survivors in the cases in question. In the one case an infiltration was observed in the subcutaneous mouse tissue, surrounded by a dense infiltrate of lymphocytes and plasma cells after 12 weeks of estrogen treatment. In the other case there was evident infiltration in the mouse's skeletal muscle, similarly surrounded by dense infiltrate of lymphocytes, with a few plasma cells and granulocytes. This mouse had received progesterone for 15 weeks. In the same case there was also an infiltration in the subcutaneous tissue of an untreated mouse after 8 weeks. In this preparation the inflammatory cell reaction was insignificant.

The mice were not autopsied and we cannot report on the possible presence of metastases. In 14 cases there were peripheral biopsies or other diagnostic material that permitted judgement of the degree of differentiation of the transplanted tumour. At least one positive transplant was retrieved in 2 of 4 well differentiated cases, in 2 of 3 moderately differentiated cases, and in 3 of 7 poorly differentiated cases. No well differentiated case proved positive after the 2nd week. The case that survived the longest (34 weeks) was poorly differentiated, but one transplant that survived for 20 weeks was moderately differentiated.

DISCUSSION

The investigation has shown that it is possible to maintain vital squamous epithelium and event to obtain continued growth for a limited period in SE in cervical biopsies from humans after implantation into nude mice. This applies both to normal epithelium and to dysplastic and carcinomatous epithelium. The method we adopted, however, has obvious limitations. It is impossible to judge the representativity of the transplanted material by investigating peripheral biopsies, since epithelial dysplasia and preinvasive cancer appear focally, and such foci can be very small. It is impossible to decide whether the atypical epithelium in a transplant has grown or regressed since the implantation. One cannot asses growth by measuring the size of the transplant as it can in varying degree evidence stroma elimination and also invasion by mouse tissue. It is often impossible to delimit exactly the transplant from the host tissue. There is also a risk that minor foci of vital epithelium can be overlooked due to shortcomings in histological technique.

Degenerative changes of varying degree will always be present in the transplant epithelium, even when it is vital and displays cell division. These degenerative changes appear erratically, which makes it exceptionally

difficult to judge any possible morphological influence of added substances or other manipulation of the experimental model.

It will be difficult to avoid transferring bacterial infection in the cervical crypts and, since the antibiotics added have presumably a low degree of penetration in this location, one must count on having an occasional transplant affected by infection, with necrosis and degenerative changes in consequence.

The overall effect of all the above will amount to difficulties in obtaining long series of well preserved transplants and that in the occasional unsuccessful transplant one will be unable to decide whether failure was due to unrepresentativity of the original material, or to destruction of epithelium in the mouse. Moreover, it is difficult to ascertain whether the morphological alterations are the result of degenerative changes or to possible manipulations of the experimental model. The large amount of stroma in this sort of biopsy is the limiting factor. If it were possible to isolate the epithelium and to be sure about the representativity of the implantation material, this model could certainly be of great value in biological studies.

ACKNOWLEDGEMENT

The excellent technical assistance of miss Elise Nilsson is gratefully acknowledged.

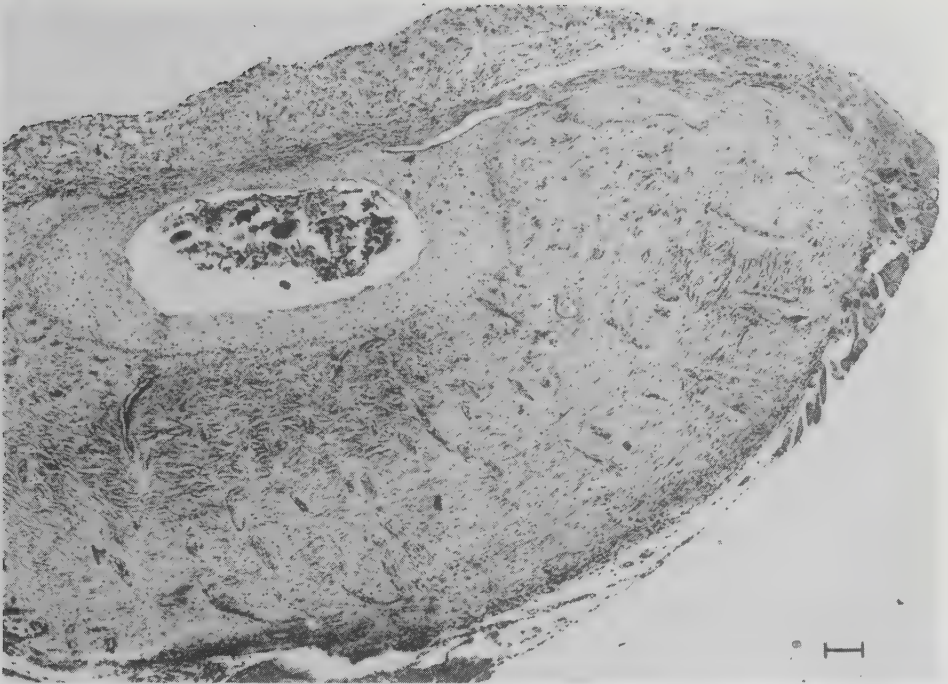


Fig. 1 Low magnification picture of transplant from normal portio 2 weeks after implantation. The stroma is partly hyalinized. The upper transplant surface is partly covered by squamous epithelium which forms an epithelium-enclosed invagination with a central cavity filled by desquamated epithelium. Subcutaneous mouse tissue (top) is seen, infiltrated by histiocytes where it is in contact with transplant epithelium. To the right and below, murine skeletal muscle can be seen. Hematoxylin-eosin. Bar equals 100 μ .

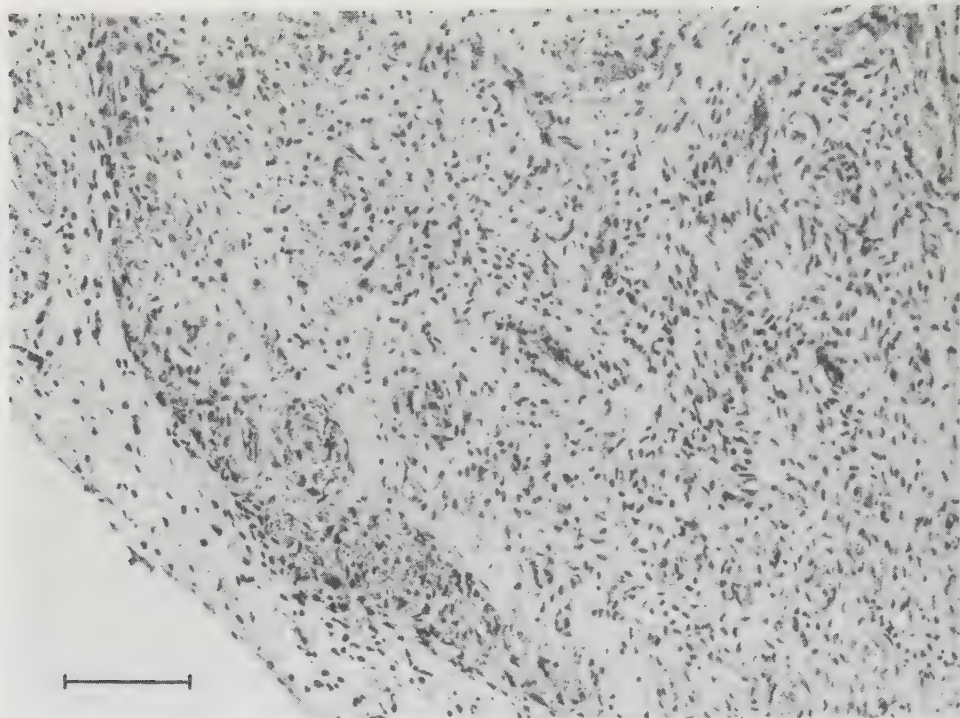


Fig. 2 Border zone between mouse tissue (below and left) and a transplant of normal portio tissue, 3 weeks after implantation. Numerous capillaries are seen invading the transplant, whose own vessels display endothelial proliferation in the lumen. Hematoxylin-eosin. Bar equals 100 μ .

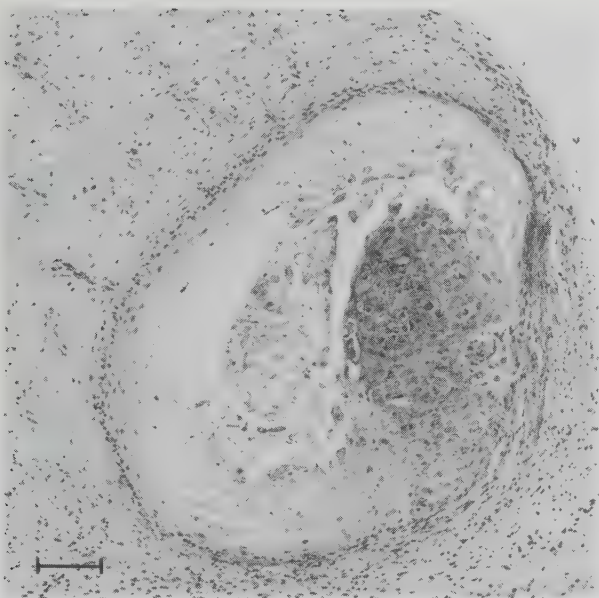


Fig. 3 Cyst with squamous epithelium in a transplant from normal portio 3 weeks after implantation. A fairly normal squamous epithelium is evident (left), but also cell vacuolization and nuclear pyknosis (below). A tangential section of epithelium is shown (right) with dyskeratotic cells. Hematoxylin-eosin. Bar equals 100 μ .

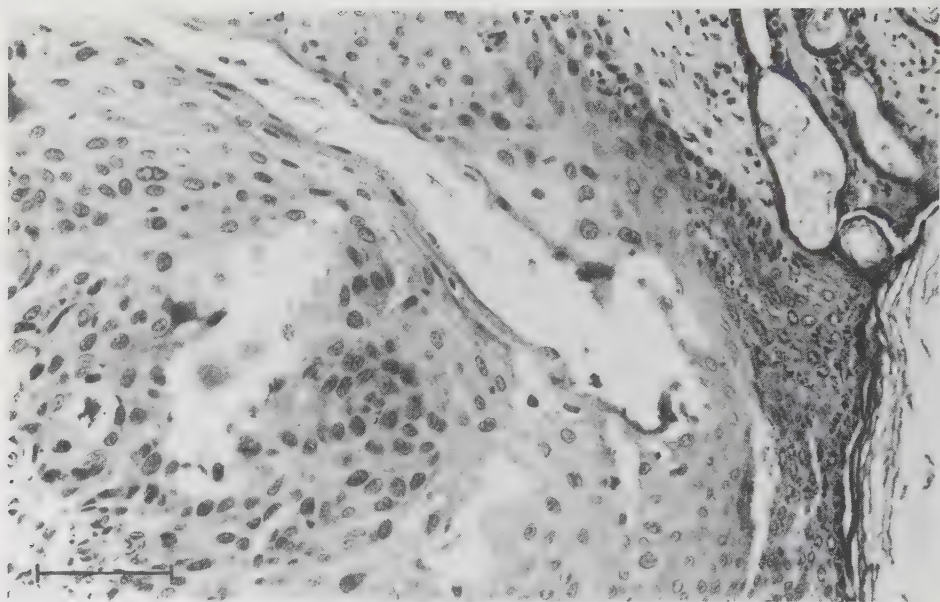


Fig. 4 A transplant with preinvasive carcinoma, 4 weeks after implantation. (Right) Mouse epidermis; (left) preinvasive cancer from the transplant; (below left) atypical mitosis; (upper right) merging of murine and transplant epithelium. Hematoxylin-eosin. Bar equals 100 μ .

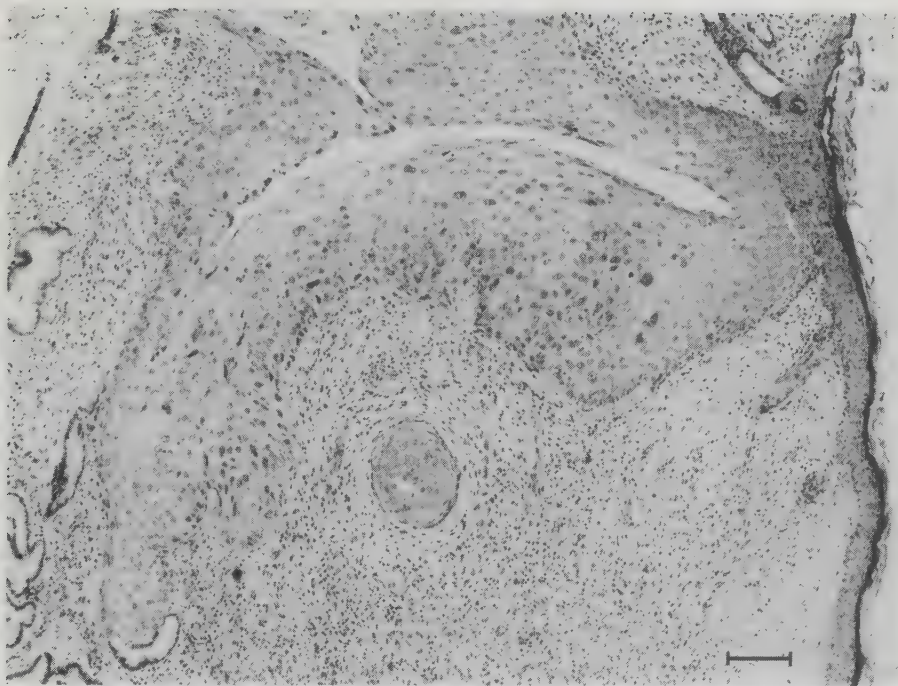


Fig. 5 Same specimens as in Fig. 4, lesser magnification. Merging between mouse dermis and transplant stroma is visible. Cervical crypts clad with columnar epithelium are also seen (left). Hematoxylin-eosin. Bar equals 100 μ .

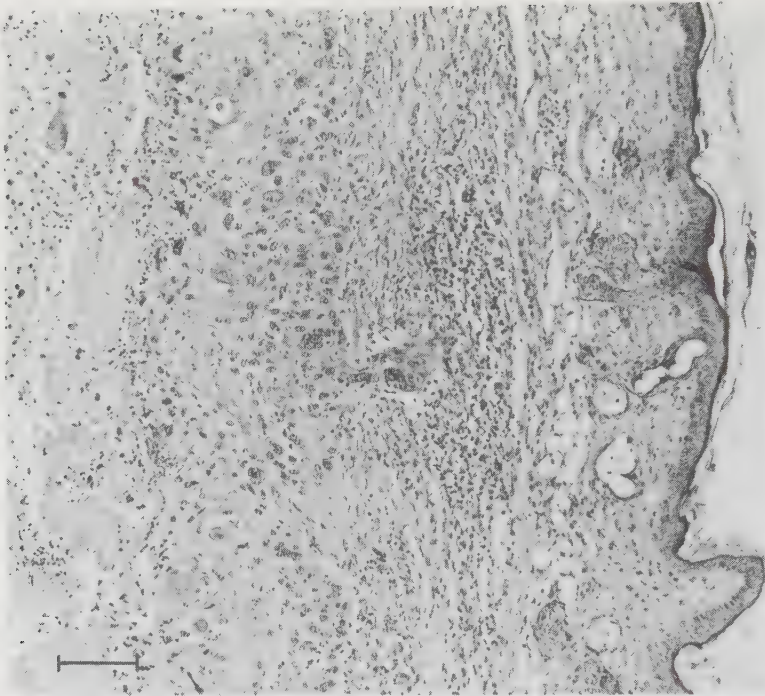


Fig. 6 Transplant with invasive carcinoma, 12 weeks after implantation. (Right) mouse skin; (left) necrotic transplant. Between the two is a zone with vital cancer. Lymphocyte infiltration can be seen in the transformation zone between the mouse tissue and the tumour. Hematoxylin-eosin. Bar equals 100 μ .

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HSV 2-ASSOCIATED ANTIGENS IN HUMAN CERVICAL TISSUES BEFORE
AND AFTER GRAFTING IN NUDE MICE

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Abstract. Biopsy material from human invasive cervical carcinoma, carcinoma in situ (CIS) and normal uterine cervix was transplanted into nude mice, some of which were then injected with estrogen or progesterone during a follow-up of 1-4 weeks. The cervical cells were analysed prior to and after passage in nude mice by the anticomplement immunofluorescence (ACIF) assay for the presence of herpes virus associated antigens. Such antigens were found in a few cells in three out of seven transplanted cervical cancers prior to the grafting and in one out of 3 cases with normal tissue. After the grafting period HSV-related antigen was found in 6 out of 7 cases of cervical cancer in grafting material from estrogen-stimulated mice. Without hormonal stimulation, 5 out of 7 showed a positive reaction. Hence the expression of the HSV-related antigen seemed enhanced after grafting in nude mice.

INTRODUCTION

Serological and epidemiological studies have suggested that herpes simplex virus (HSV) type 2 might be associated with, or one of possible causative agents in, cervical carcinoma (12,13,17,18). It is documented that women with cervical cancer have a higher incidence of neutralizing antibodies (K-test) against HSV-2 than comparable controls and react towards surface antigens of HSV-2 infected cells (2,3,4,5). It has also been shown that the antibody pattern is not the same in patients with progressing cervical lesions as in long-term survivors, in that tumor-bearing patients have low or decreasing antibody titers whereas long-term survivors have stable antibody titers towards different HSV-2 antigens (4,5,23,24). The oncogenic potential of HSV was demonstrated by the development of sarcomas in hamsters after inoculation of HSV-2 (6) and of cells transformed in vitro with HSV (16). An antigenic relationship has been detected between HSV, human cervical cancer and HSV-associated hamster tumors (9,11). The presence of HSV antigens in exfoliated cells from cervix cancer has been reported (20). It has also been revealed that normal cervical cells sometimes contain the same antigen (14).

Hormonal treatment has been shown to reduce the HSV-2-induced transformation in cell lines (8). Women with cervical cancer exhibited reduced excretions of sexual dependent steroids and had delayed menstrual cycles (10). Also, productive infection with HSV may change the receptor level at the cell thereby rendering infected cells insensitive to hormones.

This study is an attempt to 1) detect HSV associated antigens in biopsy material from cervical carcinoma, and to ascertain 2) if there are varying expressions of this antigen after passaging in nude mice, and 3) whether any influence of hormonal stimulation might be demonstrated.

MATERIAL AND METHODS

Invasive cervical cancer tissue was obtained from 5 women, one of whom proved on histological examination to have an adenocarcinoma, while the others had squamous carcinomas. Three other cervical cancer samples were of squamous CIS. Three specimens were of normal cervical tissues. The material was obtained from the Department of Obstetrics and Gynecology, General Hospital, Malmö. The tissues were obtained by excision before any treatment was initiated.

The normal cervical tissue was obtained from women of fertile age. CIS tissue was excised under kolposcopic view from fertile patients undergoing conization. Marginal pieces were taken for histological examination, thereby providing material for comparison and establishing the true nature of the material grafted. The surgical samples were cut into 1-2 mm³ pieces in medium 199 supplemented with 0.005% streptomycin sulphate and were cleansed from necrotic areas.

Four 1-2 mm³ pieces were placed subcutaneously about 1 cm apart in both flank regions of a 6-week-old female BALB-c nude mouse under ether anesthesia. Once a week one graft was removed surgically under ether anesthesia. At the final operation the animal was killed and examined for metastatic spread. Liver and lungs were inspected as well as regional lymph nodes. The absence of thymus was confirmed by inspection. Histological examination of the transplants demonstrated vital cancer cells and mitotic figures could be found.

The transplanted mice were divided into three groups. One group was given weekly injections with 0.02 mg Estradurin (Leo) subcutaneously; the second group received 5 mg Depo-Provera (The Upjohn Company); the third group served as a control. The human antiserum used in these tests was selected from a woman with invasive cervical carcinoma and with high titer against HSV-2. Control serum was also used consisting of monospecific anti sera prepared from a rabbit with HSV-2 by corneal scraping and then repeated by

three injections with virus mixed with complete Freund's adjuvant. As control cells normal cervical tissue from women of fertile age were incubated with HSV-2 and then transplanted into nude mice in the same way as the cervical cancer tissue fragments. After the last transplants were taken the animals were killed and when examined, HSV-2 could be isolated from the brain.

The transplanted tissue fragments infected with HSV-2 were examined and handled in exactly the same way as the invasive cervical cancer tissue.

Anticomplement immunofluorescence (ACIF) test. The tissue was minced and washed for three 10-min periods in PBS containing 1% inactivated fetal calf serum (FCS). Two drops of minced tissue were placed on a slide on two ring spots, so that the same slide could be tested with both positive and negative sera. Five to twenty slides were prepared from each specimen. They were dried at room temperature and fixed for 5 min in an equal volume of chilled acetone-methanol (-20°C). The slides were kept at -70°C until used. A human serum that gave no reaction in the antibody-dependent cell-mediated cytotoxicity (ADCC) assay (21,22) and which was negative in conventional neutralization test, was used as a complement source and as the non-immune HSV serum.

Sera were inactivated at 56°C for 30 min prior to use. Before staining, the slides were dropped with guinea pig complement ($1.5\ \mu\text{l/ml}$) incubated at 37°C for 20 min, after which they were washed with stirring for 30 min. The cells were stained with a suitable dilution of FITC-conjugated anti-human B1C/B1A globulin (Hyland Laboratories, Los Angeles, Calif., USA). The ACIF technique used by Reedman & Klein (19) for the detection of Epstein-Barr virus antigens was adopted, with some modification. The slides were coded and read blind. Thin sectioning of the tissue transplants was also tried but this method gave more non-specific staining than when using minced tissue.

Controls to test the specificity of the reagents used, HSV-2 infected and uninfected GMK-cells, were stained with the ACIF assay and by the indirect immunofluorescence technique. The GMK cells were incubated with high-multiplicity dose of HSV-2, incubated overnight and harvested before any CPE could be detected macroscopically.

Conventional neutralization test (NT). Briefly, 2-fold dilutions of serum were mixed with equal volumes of a virus suspension containing 100 TCID₅₀ per 0.1 ml of HSV type 2. The mixtures were allowed to stand for 2 h at room temperature and were assayed on tube culture of GMK-AH1 cells. Each mixture was inoculated into five culture tubes at a volume of 0.2 ml per tube. The endpoint titer was read 7 days after inoculation.

Kinetic neutralization test (K-test). By utilizing the kinetics of neutralization, high avidity antibodies against HSV types 1 and 2 can be calculated. The important property of this method is that it stops the reaction between serum antibody and virions after a short contact period.

The original technique was followed exactly (5,17,18). Briefly, the test was performed by adding HSV type 1 or type 2 virus to a dilution of serum. The virus-serum mixtures were held at 37°C for 2 min. A mixture was then removed, diluted in cold diluent and assayed for surviving virus.

Antibody-dependent cell-mediated cytotoxicity (ADCC). The method was performed as previously described (3,21, 22). HSV-2-infected and uninfected GMK cells were labelled with Na₂⁵¹CrO₄. Labelled cells (target cells) together with lymphocytes (effector cells) and dilutions of test serum were incubated for 4 hours. After inoculation the isotope release was determined. Augmentation of cytotoxicity above background was expressed as the percentage of ⁵¹Cr release observed with lymphocytes and test sera minus the percentage release with lymphocytes alone.

Mixed hemadsorption (MH) test. The culture technique, virus infection, and assay for antibody reaction to HSV-2-induced surface antigens have been described in a previous paper (4). Briefly, bottles with confluent GMK cells were inoculated with HSV-2. After incubation, the bottles were overlaid with agar. The sera to be tested were applied on filter paper discs. After further incubation at room temperature, the agar layer was removed and indicator red cells were added.

RESULTS

In order to test the specificity of the system used in this survey, HSV-2-infected and uninfected GMK cells were stained with human positive and negative HSV-2 sera. Control cells also consisted of normal cervical tissue infected with HSV-2 prior to transplantation into nude mice. Cells from these transplants demonstrated typical HSV fluorescence and HSV-2 could be isolated from the brain of the mice. Fig. 1(A) demonstrated reactivity of HSV-2 in 12-hour post-infected GMK cells obtained with the ACIF assay using the human HSV-2 positive serum from a woman with invasive cervical cancer. Some 5-20 slides from each tissue sample before or after grafting were made, each slide being tested with positive and negative human HSV serum. Fig. 1(B) shows squamous carcinoma cells after a 4-week passage in a nude mouse. Results were considered positive if the cells displayed positive fluorescence with anti-HSV serum but not with negative HSV-serum. Antigen-positive cells displayed either nuclear, perinuclear, cytoplasmic, or whole-cell fluorescence. Many cells showed only membrane fluorescence; these cells were considered non-specific as Fc-receptors not could be precluded. There were several cells, from both normal and cancerous tissues, which gave non-specific reactions with both positive and negative human HSV-sera, though not sera positive or negative stained uninfected GMK cells.

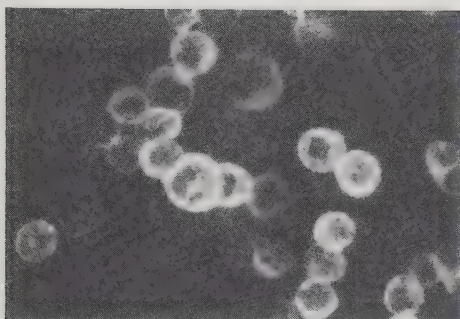


Fig. 1(A) HSV-2 infected GMK cells. Twelve hours postinfected, stained by the ACIF technique

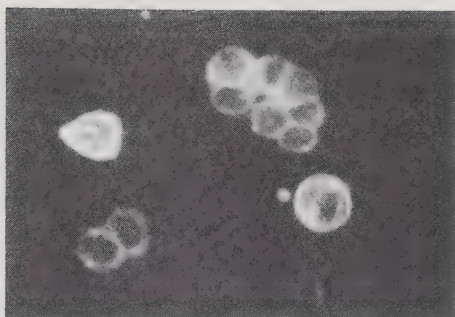


Fig. 1(B) ACIF-stained cervical carcinoma cells after passage in nude mouse which had been given weekly injections with Estradurin

Sera were obtained prior to any kind of treatment at the time of biopsy or surgery. All women, both those with squamous cancer and CIS and those with normal tissues, had experienced a past HSV infection (Table I). All showed seropositivity on conventional neutralization test and all demonstrated positive reaction in the antibody-dependent cell-mediated cytotoxicity (ADCC) assay, i.e. augmented cytotoxicity statistically different from values obtained with lymphocytes alone (3,21,22). The ADCC assay is useful for detecting minute amounts of HSV antibodies in sera otherwise negative in conventional tests, thus reflecting a past HSV infection. Two women with normal cervical tissue showed specific K_1 activity. One also had K_2 activity ($K_2 > 4$). Five out of the 8 women with a cancerous lesion had specific K_2 activity.

Table II gives the results with the ACIF test using human anti-HSV-2 sera. In 3 out of 8 tissues from women with squamous cervical carcinoma and CIS a small number of positive cells were found prior to transplantation. After

transplantation into nude mice, HSV-associated antigens were detected in 6 out of 7 transplants in the group that had been given estrogen inoculation. In the progesterone group, 3 out of 7 transplants were positive and in the mouse-passage group which served as a control, 5 out of 7 had a positive reaction. In one out of 3 transplants from normal cervical tissue, positive fluorescence was detected prior to transplantation. After mouse-passage and estrogen stimulation, a further one had a positive reaction. None of the three in the progesterone group was positive.

DISCUSSION

When using the anticomplement immunofluorescence technique, a few cells of the cervical cancers tested demonstrated a positive reaction with human HSV-2 sera. The results suggest that there might be an HSV-associated antigen in cells obtained from cervical carcinoma tissue. An antigenic relationship has been demonstrated previously between HSV, human cervical cancer and HSV-associated hamster tumors by means of the ACIF assay (11).

It has been reported that, by using the indirect immunofluorescence assay, HSV antigens could be detected in exfoliated cervical neoplastic cells, but not directly in cervical neoplastic tissue (20). HSV antigens have also been reported with the indirect immunofluorescence technique in women with normal cervical epithelium (14) and to a great extent in women with invasive cervical cancer. Contrary to these findings we were unable to find any HSV-related antigen by the indirect immunofluorescence test. A small number of fluorescent positive cells were found in specimens from 3 out of 7 cervical cancer patients with squamous cervical cancer and CIS and in 6 out of 7 after mouse-passaging and estrogen stimulation.

In one case out of 3 with normal cervical tissue, positive fluorescence was found prior to transplantation and in 2 of these cases after mouse passage and estrogen injections.

The number of positive cells and the intensity of the immunofluorescence seemed enhanced after passaging the material in nude mice and this was particularly marked in material from mice that were given estrogen injections. Due to wastage of mice during the follow-up it was difficult, however, to distinguish with certainty between transplants from Estradurin- and Depo-Provera-treated mice or mouse-passaged transplants without hormonal injections.

The main problem in this material concerns the significance of the fluorescence demonstrated. Is it an antigen which is associated with a cancer-related HSV antigen, or is it merely the fingerprint of a past HSV infection with no relation to cervical cancer? Cancerous cells might be a favorable tissue environment for harboring HSV. On the other hand, non-cytopathogenic cell-associated virus is capable of transforming cells in vitro (7,16). A substantial number of cells stained non-specifically. This might have many explanations: it might be due to complement and Fc-receptors, or to heterophil antibodies in human sera against various tissue structures on cervical cells.

The significance of sex-related steroids for the development of cervical cancer is not yet clarified, but a number of steroidal deficiencies have been demonstrated in women with cervical cancer, such that premenopausal women have a reduced excretion of androgen and progestin which may affect the expressions of estrogenic action on the target cell.

Following protracted administration of estrogen, cervical cancer has been produced in mice. In monkey, lesions were induced which were considered precancerous but failed to produce cervical cancer (1). It has also been demonstrated that hormonal treatment of a mouse cell line reduced the HSV-2-induced transformation (8). The significance of sex-related steroids for the development of cervical cancer has not been elucidated, but there have been no reports of any relationship between progesterone and cervical carcinoma.

One may speculate that certain strains of HSV might be oncogenic under certain conditions such as hormonal imbalance or changes in the receptor levels in the cell. Excessive stimulation as well as understimulation might change the normal rejection of foreign antigens such that forbidden cell clones might be allowed to grow. It is quite conceivable the hormonal imbalance favors the development of malignant transformation. The expression of HSV-related antigens may differ, as may the ability to detect this antigen in cancerous cells. It seemed as if the expression of the HSV-related antigens was enhanced after passage in nude mice and especially in those mice which were subjected to estrogen stimulation. The restrictive influence of the human host on the production or expression of certain antigens may be lost in the mouse host (15). Such stimulation might favor the persistent expression of HSV-2-related antigens in biopsy material during passage in nude mice. It is difficult to draw any general conclusions, however, as the patients tested were few and there was a wastage of mice during the follow-up.

However, this study shows that cervical cells both from cancerous tissue and from normal tissue contain antigens related to those found in cells infected with HSV-2 and that the expression of these antigens might be favored by sex-hormone stimulation and/or passage in nude mice. These findings by no means answer the question of whether cervical neoplasia originates from antigen-carrying cells. The association of neoplasia and HSV-2 could be based on a co-factoral role of the virus.

ACKNOWLEDGEMENT

The technical assistance of Miss Lisbeth Henriksson is gratefully acknowledged.

ABBREVIATIONS

ADCC: antibody-dependent cell-mediated cytotoxicity; ACIF: anticomplement immunofluorescence; CF: complement fixation; CIS: carcinoma in situ; CPE: cytopathic effect; FCS: fetal calf serum; GMK: green monkey kidney; HSV: herpes simplex virus; C: constant of virus inactivation by neutralizing antibodies; MC: mononuclear cells; MH: mixed hemadsorption.

kinetic neutralization test (K-test), to HSV-2 infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) and mixed hemadsorption (MH) assays, and by complement-fixation (CF) test

Cell type tested	NT	K-values		ADCC* % augmented cytotoxicity	MH**	CF
		K ₁	K ₂			
Cancer in situ						
M 7	128	5.5	5.1	5.8 ⁺ 1.0 p<0.01	10	80
M 10	293	7.4	14.7	16.3 ⁺ 1.1 p<0.001	15	80
M 17	14	<4	4.3	3.6 ⁺ 0.3 p<0.01	-	5
Invasive cervical cancer						
M 11	2	<4	<4	3.5 ⁺ 0.5 p<0.01	-	5
M 12	56	<4	9.2	18.9 ⁺ 3.0 p<0.001	not done	20
M 14	10	7.4	<4	10.1 ⁺ 2.0 p<0.01	15	20
M 15	32	11.0	4.1	28.4 ⁺ 3.9 p<0.001	15	40
Adenocarcinoma						
M 9	97	4.1	<4	5.3 ⁺ 1.5 p<0.01	15	20
Normal cervical cells						
M 6	32	4.1	<4	6.1 ⁺ 1.8 p<0.01	10	10
M 8	8	<4	<4	4.0 ⁺ 1.2 p<0.01	-	10
M 16	128	14.7	7.4	18.6 ⁺ 1.5 p<0.001	15	40

* Augmented cytotoxicity: percentage ⁵¹Cr release obtained with lymphocytes and test sera minus the percentage of ⁵¹Cr release obtained with lymphocytes and test

Table II Reaction observed with anticomplement immunofluorescence (ACID) assay to human cervical cancer and to normal cervical tissues after passage in nude mice, some injected with either estrogen or progesterone

Cell type tested	0 ^{a)}	Estrogen in-				Progesterone				Passage in			
		jected (weeks)				injected (weeks)				control mice (weeks)			
		1	2	3	4	1	2	3	4	1	2	3	4
Cancer in situ													
M 7	+	+	+	-	-	+	+	+	+	+	0	-	-
M 10	+	+	0	0	+	0	+	0	0	+	0	0	0
M 17	0	0	-	-	-	0	0	0	-	0	-	-	+
Squamous cervical cancer													
M 11	0	+	0	+	-	-	-	-	-	0	0	0	-
M 12	+	+	-	-	-	0	0	-	-	0	-	-	-
M 14	0	0	0	+	-	+	0	+	0	+	0	0	-
M 15	0	+	+	+	-	0	-	-	-	0	0	+	0
Adenocarcinoma													
M 9	0	0	0	0	-	0	0	0	-	0	0	0	-
Normal cervical tissue													
M 6	0	0	-	+	-	0	0	0	-	0	0	-	-
M 8	0	0	0	0	-	0	0	-	-	0	-	-	-
M 16	+	0	+	-	-	0	0	-	-	0	+	-	-

A) prior to implantation b) + = positive; 0 = negative; - = not tested

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